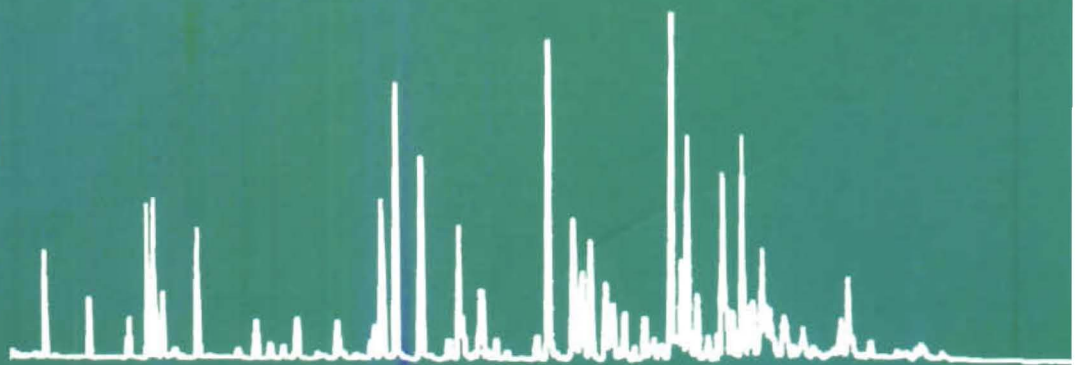


The Essence of Chromatography

Colin F. Poole



Elsevier

The essence of chromatography

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Preface

The knowledge base of chromatography continued to expand throughout the 1990s owing to its many applications to problems of contemporary interest in industry and life and environmental sciences. Organizing this information into a single text for a diverse group of scientists has become increasingly difficult. The present book stemmed from the desire to revise an earlier work, "Chromatography Today", published in 1991. It was soon realized that a simple revision would not provide the desired result of a contemporary picture of the practice of chromatography at the turn of the century. The only workable solution was to start afresh, maintaining the same general philosophy and concept for "Chromatography Today" where possible, while creating essentially a new book. In particular, both time and space constraints dictated that to cover in equal depth the diverse separation techniques in current use, it would not be possible to cover sample preparation techniques to the same extent as "Chromatography Today". The division I made here was to include automated and on-line methods with instrumentation, and treat them in a comprehensive manner, while widely used manual laboratory operations are not treated at all, albeit that these techniques are an integral part of laboratory life. This allowed, for example, the addition of a comprehensive and separate chapter on capillary-electromigration separation techniques, and greater emphasis on modern approaches for data analysis, compared with "Chromatography Today".

In writing this book, I had in mind that it would present a comprehensive survey of modern chromatographic and capillary electrophoretic techniques at a level commensurate with the needs of a textbook for teaching post-baccalaureate courses in the separation sciences. In addition, it would fulfill the need for a self-study guide for professional scientists wishing to refresh their background in this rapidly growing field. The chapters follow a modular format to allow instructors to select components to their liking to make up a typical one-semester course. For the professional scientist, the extensive cross-referencing and comprehensive index should allow individual topics to be quickly found, and the extensive bibliography to be used for entry into the primary scientific literature. Where possible, frequently searched for characteristic properties of separation systems are collected in tables, to allow the book to be used as a stand-alone resource for the professional scientist.

Colin F. Poole

Chapter 1

General Concepts in Column Chromatography

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1.1 INTRODUCTION

The Russian botanist M. S. Tswett is generally credited with the discovery of chromatography around the turn of the century [1,2]. He used a column of powdered calcium carbonate to separate green leaf pigments into a series of colored bands by allowing a solvent to percolate through the column bed. He also coined the name chromatography (color writing) from the Greek for color (chroma) and write (graphein) to describe the process. However, column liquid chromatography as described by Tswett was not an instant success, and it was not until its rediscovery in the early 1930s that it became an established laboratory method. Chemists at this time were limited to such laboratory tools as crystallization, liquid-liquid distribution and distillation for separations and new techniques were needed for the rapid isolation of pure components from natural products and to support the development of increasingly sophisticated approaches to organic synthesis. Although many scientists made substantial contributions to the evolution of modern chromatography, not least among these is A. J. P. Martin who received the Nobel prize in 1952 for the invention of partition chromatography (with R. L. M. Synge) and in the same year with A. T. James he introduced the technique of gas-liquid chromatography. The 1940s saw a rapid expansion in the use of chromatographic methods in the laboratory but the introduction and development of gas-liquid chromatography in the 1950s represented a significant milestone, ushering in the era of instrumental methods of separation which spawned the many variations of modern chromatography in use today. Further milestones in the evolution of chromatographic separation methods are summarized in Table 1 [3-5]. Individual profiles of the early pioneers of chromatography are collected in ref. [6-8].

1.2 FAMILY TREE OF CHROMATOGRAPHIC METHODS

Since chromatography has evolved into a large number of applied methods it is no simple task to provide a meaningful comprehensive definition. Chromatography is essentially a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction [9,10]. This definition suggests that chromatographic separations have three distinct features: (a) they are physical methods of separation; (b) two distinct phases are involved, one of which is stationary while the other is mobile; and (c) separation results from differences in the distribution constants of the individual sample components between the two phases. The definition could be broadened to allow for the fact that it is not essential that one phase is stationary, although this may be more experimentally convenient. What is important, is that either the rate of migration or direction of migration of the two phases are different [11]. Micellar electrokinetic chromatography (MEKC) is an example of a separation technique based on differential migration in a two-phase system. The above definition excludes all separations that occur by differential migration in a

Table 1.1

Some significant time frames in the evolution of modern chromatography

Date	Associated development
1903	<ul style="list-style-type: none"> • Original description of column liquid chromatography by Tswett
1931	<ul style="list-style-type: none"> • Column liquid chromatography rediscovered by Lederer and co-workers at a time more receptive for its establishment as a standard laboratory method.
1938	<ul style="list-style-type: none"> • Ion-exchange column liquid chromatography introduced. It came to prominence as a distinct chromatographic technique during the Second World War (1939-1945) as a separation procedure for the rare earth and transuranium elements.
1941	<ul style="list-style-type: none"> • Column liquid-liquid partition chromatography introduced as a faster and more efficient separation method than countercurrent distribution chromatography.
1944	<ul style="list-style-type: none"> • Paper chromatography introduced as a fast, simple and convenient method for analytical separations based on partition chromatography. Now largely replaced by thin-layer chromatography.
Mid-1940s	<ul style="list-style-type: none"> • Gel electrophoresis developed for the separation of charged analytes in a stabilizing gel matrix. Later became an important method for the separation of biopolymers.
Early-1950s	<ul style="list-style-type: none"> • Immobilized layers and standardized sorbents leads to the popularization of thin-layer chromatography as a faster and more convenient method than column liquid chromatography for analytical separations. Fine-particle layers introduced in the mid-1970s were responsible for the development of high performance (instrumental) thin-layer chromatography.
1952	<ul style="list-style-type: none"> • Gas-liquid chromatography is described by James and Martin and begins the development of instrumental chromatographic methods. Gas chromatography provided a major improvement in the separation of volatile compounds eclipsing established methods at that time. It remains the most widely used chromatographic technique for the fast and efficient separation of thermally stable and volatile compounds today.
1958	<ul style="list-style-type: none"> • Column liquid size-exclusion chromatography using controlled porosity dextran gels introduced by Flodin and Porath. This became an important approach for the separation (or characterization) of polymers based on size differences as well as for the estimation of molecular weights.
1962	<ul style="list-style-type: none"> • Klesper introduced supercritical fluids as mobile phase for column chromatography but limited development took place until the early 1980s when Lee introduced open tubular columns. Most supercritical fluid separations today use packed columns of small internal diameter.
Mid-1960s	<ul style="list-style-type: none"> • Giddings introduces the technique of field flow fractionation for the separation of particles and continues to develop the theory and technology of its many variants (fields) over the next 30 years.
1967	<ul style="list-style-type: none"> • Affinity chromatography introduced by Porath and co-workers for the isolation of biological polymers based on the specificity of their interactions with appropriate immobilized ligands.
Late-1960s	<ul style="list-style-type: none"> • The introduction of pellicular sorbents catalyzed the development of high pressure liquid chromatography. It was not until the mid-1970s that rapid development took place with the introduction of porous microparticle sorbents. By the 1980s high pressure liquid chromatography was well established as the most popular condensed phase separation technique in modern chromatography.
1970	<ul style="list-style-type: none"> • Everaerts and co-workers introduced capillary isotachopheresis for the concentration and separation of ions.
1970s	<ul style="list-style-type: none"> • Ito and co-workers commenced a number of advances in counter current chromatography using centrifugal and planetary motion for liquid-liquid separations.
Mid-1970s	<ul style="list-style-type: none"> • Small and co-workers introduced ion chromatography based on the integration of ion-exchange chromatography with conductivity detection for the analysis of ions. This method is now the most common chromatographic technique for the analysis of inorganic ions.

Table 1.1
(Continued)

Date	Associated development
Early-1980s	<ul style="list-style-type: none"> ● Jorgenson and co-workers popularized the use of zone electrophoresis in capillary columns for the fast and efficient separation of ions and biopolymers.
1984	<ul style="list-style-type: none"> ● Terabe introduced the method of micellar electrokinetic chromatography (MEKC) using surfactant-containing buffers in a capillary electrophoresis apparatus. Over the next decade MEKC matured into an important method for the electroseparation of neutral compounds.
Late-1980s	<ul style="list-style-type: none"> ● Rediscovery of capillary electrochromatography. Pioneering work by Knox leads to the evolutionary development of this technique during the 1990s.

single-phase system, such as capillary electrophoresis (CE). Useful chromatographic separations require an adequate difference in the strength of physical interactions for the sample components in the two phases, combined with a favorable contribution from system transport properties that control the movement within and between phases. Several key factors are responsible, therefore, or act together, to produce an acceptable separation. Individual compounds are distinguished by their ability to participate in common intermolecular interactions in the two phases, which can generally be characterized by an equilibrium constant, and is thus a property predicted from chemical thermodynamics. During transport through or over the stationary phase differential transport resulting from diffusion, convection, turbulence, etc., result in dispersion of solute zones around an average value, such that they occupy a finite distance along the stationary phase in the direction of migration. The extent of dispersion restricts the capacity of the chromatographic system to separate, and independently of favorable thermodynamic contributions to the separation, there are a finite number of dispersed zones that can be accommodated in the separation. Consequently, chromatographic separations depend on a favorable contribution from thermodynamic and kinetic properties of the compounds to be separated.

A convenient classification of chromatographic techniques can be made in terms of the physical state of the phases employed for the separation, Figure 1.1. When the mobile phase is a gas and the stationary phase a solid or liquid the separation techniques are known as gas-solid chromatography (GSC) or gas-liquid chromatography (GLC), respectively. Gas-liquid chromatography is the more popular separation mode and is often simply referred to as gas chromatography (GC). When the mobile phase is a supercritical fluid and the stationary phase either a solid or immobilized liquid the separation technique is called supercritical fluid chromatography (SFC). For gas and supercritical fluid chromatography the dominant separation mechanisms are partitioning between bulk phases and interfacial adsorption. To classify separation techniques with a liquid mobile phase a wider range of separation mechanisms needs to be considered and is commonly used as the basis of classification. Also, true liquid-liquid separation systems are not important because of their limited stability and experimental inconvenience. Modern liquid chromatography is dominated by the use of inorganic oxides with organic functional groups chemically bonded to their surface, known as bonded phases, and to a lesser extent porous polymers. When the stationary phase is

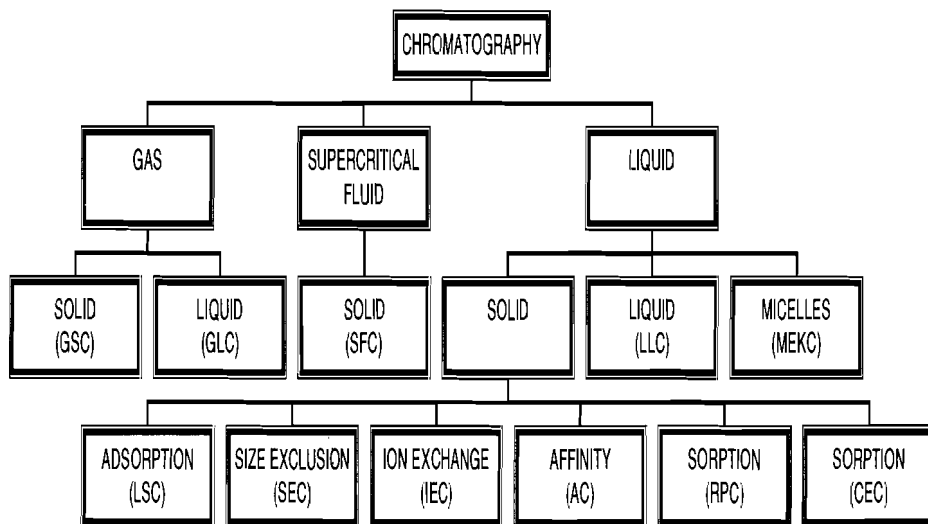


Figure 1.1. Family tree of column chromatographic methods. GSC = gas-solid chromatography; GLC = gas-liquid chromatography; SFC = supercritical fluid chromatography; LLC = liquid-liquid chromatography; MEKC = micellar electrokinetic chromatography; LSC = liquid-solid chromatography; SEC = size-exclusion chromatography; IEC = ion-exchange chromatography; AC = affinity chromatography; RPC = reversed-phase chromatography; and CEC = capillary electrochromatography.

a solid and interfacial adsorption the dominant separation mechanism the technique is referred to as liquid-solid chromatography (LSC). If the stationary phase is a solid with a controlled pore size distribution and solutes are separated by size differences then the technique is referred to as size-exclusion chromatography (SEC). If the stationary phase is a solid with immobilized ionic groups and the dominant separation mechanism is electrostatic interactions between ions in the mobile phase and those on the stationary phase then the technique is referred to as ion-exchange chromatography (IEC) or ion chromatography (IC). If the stationary phase is a solid with immobilized molecular recognition sites in which the dominant separation mechanism is the three-dimensional specificity of the interaction between the molecular recognition site and the sample then the technique is referred to as affinity chromatography (AC). Reversed-phase chromatography (RPC) is a particular form of bonded-phase chromatography in which the mobile phase is more polar than the stationary phase (for most practical applications the mobile phase is an aqueous solution). Reversed-phase chromatography is the most popular separation mode in modern liquid chromatography being applicable to a wide range of neutral compounds of different polarity. In addition, by exploiting secondary chemical equilibria in the mobile phase, ionic compounds are easily handled by ion suppression, ion pairing, or complexation.

In the normal operating mode in gas, supercritical fluid and liquid chromatography the stationary phase is contained in a rigid container, usually a tube of various

dimensions, called a column, through which the mobile phase is forced to migrate by external pressure. Alternatively, the bulk flow of mobile phase containing an electrolyte can be induced by an external electric field through the process known as electroosmosis. When a column containing a stationary phase is used and the movement of the mobile phase is caused by electroosmosis the separation technique is called electrochromatography, or since columns of capillary dimensions are essential for this technique, capillary electrochromatography (CEC). Ionic surfactants can form micelles as a continuous phase dispersed throughout a buffer. In an electric field these charged micelles move with a different velocity or direction to the flow of bulk electrolyte. Neutral solutes can be separated if their distribution constants between the micelles and buffer are different by the technique known as micellar electrokinetic chromatography (MEKC). Ionic solutes in CEC and MEKC are influenced by the presence of the electric field and are separated by a combination of chromatography and electrophoresis. All the above processes are considered examples of column chromatography. If the stationary phase is distributed as a thin layer on a (usually) flat support, such as a sheet of glass or plastic, and the mobile phase is allowed to ascend through the layer (usually) by capillary forces then this method is referred to as planar or thin-layer chromatography (TLC). TLC has largely replaced paper chromatography (PC) in contemporary practice owing to the poorer separation characteristics of the latter.

1.3 ZONE MIGRATION

Transport of solute zones in column chromatography occurs entirely in the mobile phase. Transport is an essential component of the chromatographic system since the common arrangement for the experiment employs a sample inlet and detector at opposite ends of the column with sample introduction and detection occurring in the mobile phase. There are three basic approaches for achieving selective zone migration in column chromatography, Figure 1.2 [12]. In frontal analysis, the sample is introduced continuously onto the column as a component of the mobile phase. Each solute is retained to a different extent as it reaches equilibrium with the stationary phase until, eventually, the least retained solute exits the column followed by other zones in turn, each of which contains several components identical to the solutes in the zone eluting before it [13]. Ideally the detector output will be comprised of a series of rectangular steps of increasing height. Frontal analysis is used to determine sorption isotherms for single component or simple mixtures and to isolate a less strongly retained trace component from a major component. Quantification of each component in a mixture is difficult and at the end of the experiment, the column is contaminated by the sample. For these reasons frontal analysis is used only occasionally for separations. Frontal analysis is the basis of solid-phase extraction techniques used for the collection of contaminants from air and water by sorption onto short sorbent beds.

In displacement chromatography the sample is applied to the column as a discrete band and a substance (or mobile phase component) with a higher affinity for

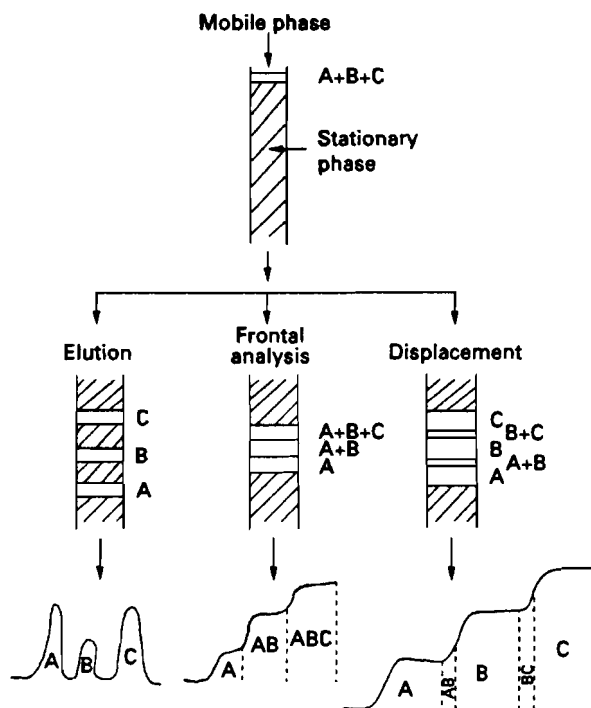


Figure 1.2. Mode of zone displacement in column chromatography.

the stationary phase than any of the sample components, called the displacer, is continuously passed through the column. The displacer pushes sample components down the column, and if the column is long enough, a steady state is reached, and a succession of rectangular bands of pure components exit the column. Each component displaces the component ahead of it, with the last and most strongly retained component being forced along by the displacer. At the end of the separation the displacer must be stripped from the column if the column is to be reused. Displacement chromatography is used mainly in preparative and process chromatography, where high throughputs of pure compounds can be obtained (section 11.3.5) [12]. Depending on the experimental conditions the contact boundary between zones may not be discrete and the collection of pure material may be restricted to the central region of the displaced zones.

In elution chromatography the mobile and stationary phase are normally at equilibrium. The sample is applied to the column as a discrete band and sample components are successively eluted from the column diluted by mobile phase. The mobile phase must compete with the stationary phase for the sample components and for a separation to occur the distribution constants for the sample components resulting from the competition must be different. Elution chromatography is the most convenient method for analysis and is commonly used in preparative-scale chromatography.

Today elution development has become synonymous with the word chromatography itself.

The information obtained from a chromatographic experiment is contained in the chromatogram. When the elution mode is used this consists of a plot of (usually) detector response (y-axis) as a continuous function of time or volume of mobile phase passed through the column (x-axis). The chromatogram contains a number of peaks of various sizes rising from a baseline. Many representative examples can be found throughout this text. Information readily extracted from the chromatogram includes an indication of sample complexity from the number of observed peaks; qualitative identification of sample components from the accurate determination of peak position; quantitative assessment of the relative concentration or amount of each component from their peak areas; and characteristic physical properties of either the solute or the chromatographic system from peak positions and profiles. The fundamental information of the chromatographic process that can be extracted from the chromatogram forms basis of the remainder of this chapter.

1.4 RETENTION

The position of a peak in a chromatogram is characterized by its retention time (t_R) or retention volume (V_R). Retention volumes are fundamentally more correct than time but require further experimental information for their determination. We will come to this shortly, and consider only the directly observable measurement of time for the present. The retention time is made up of two components. The time that the solute spends in the mobile phase and the time it spends in the stationary phase. All solutes spend the same time in the mobile phase, which is simply the time required by an unretained solute, that is a solute that does not interact with the stationary phase, to travel through the chromatographic system. This time is called the column hold-up time, t_M , (sometimes referred to as the dead time although hold-up time is preferred). It represents the time required by the mobile phase entering the column to reach the detector and in volume terms is equivalent to the volume of streaming mobile phase contained in the column. In liquid and supercritical fluid chromatography a fraction of the mobile phase can be trapped in the pores of the column packing and is stagnant. The volume of stagnant mobile phase is considered part of the stationary phase and thus the column hold-up volume is less than the volume of liquid or fluid filling the column. For a gas the column hold-up volume and the unoccupied volume of the column are identical. The time the solute spends in the stationary phase is called the adjusted retention time, t_R' (or adjusted retention volume, V_R') and is calculated by difference from the retention time (volume) and the column hold-up time (volume). Since for convenience the retention time of a substance is determined from the moment of injection as time zero, we arrive at the simple relationship (Eq. 1.1) combining the independent contributions to the observed retention time

$$t_R = t_M + t_R' \quad (1.1)$$

For the optimization of chromatographic separations and in the formulation of theoretical models the retention factor (sometimes referred to as the capacity factor), k , is more important than retention time. The retention factor is the ratio of the time a substance spends in the stationary phase to the time it spends in the mobile phase (Eq. 1.2)

$$k = t_R' / t_M = (t_R - t_M) / t_M \quad (1.2)$$

If the distribution constant is independent of the sample amount then the retention factor is also equal to the ratio of the amounts of substance in the stationary and mobile phases. At equilibrium the instantaneous fraction of a substance contained in the mobile phase is $1 / (1 + k)$ and in the stationary phase $k / (1 + k)$. The retention time and the retention factor are also related through Eq. (1.3)

$$t_R = t_M (1 + k) = (L / u) (1 + k) \quad (1.3)$$

where L is the column length, and u the average mobile phase velocity. The distribution constant for a substance in the chromatographic system is equal to the product of the retention factor and the phase ratio ($K = k\beta$). The phase ratio, β , is defined as the ratio of the volume of mobile phase and stationary phases in the column for a partition system, or the ratio of the volume of the mobile phase to the surface area of the stationary phase for an adsorption system, respectively.

The relative retention of any two peaks in the chromatogram is described by the separation factor, α , given by Eq. (1.4)

$$\alpha = t_R'(B) / t_R'(A) = k_B / k_A \quad (1.4)$$

By convention, the adjusted retention time or retention factor of the later eluting of the two peaks is made the numerator in Eq. (1.4); the separation factor, consequently, always has values greater than or equal to 1.0. The separation factor is a measure of the selectivity of a chromatographic system. In thermodynamic terms it is related to the difference in free energy of the retention property responsible for the separation and is a term widely used in method development for defining systems with useful separation properties. To maintain a useful thermodynamic meaning the separation factor must be determined for fixed and constant experimental conditions, for example, constant temperature in gas chromatography and constant mobile phase composition in liquid chromatography. The separation factor is sometimes called the selectivity factor, selectivity or relative retention.

1.4.1 Influence of Mobile Phase Physical Properties

In pressure-driven systems a pressure gradient exists between the column inlet and outlet resulting in a change in volume-dependent terms over the length of the column

Table 1.2

Calculation of retention volumes in gas chromatography

Experimental data for calculation: retention time $t_R = 12.61$ min; column hold-up time $t_M = 0.23$ min; carrier gas flow rate at column outlet $F_a = 21.78$ ml/min; column temperature $T_c = 121^\circ\text{C}$ (394.2 K); ambient temperature $T_a = 23^\circ\text{C}$ (296.2 K); ambient pressure $P_0 = 754.5$ mm Hg; column head pressure $P_G = 62.9$ mm Hg; P_w = vapor pressure of water at T_a (available in handbooks of physical constants); and weight of liquid phase $W_L = 1.5115$ g

• Calculation of the gas compressibility correction factor j

$j = 3 / 2 [(P^2 - 1) / (P^3 - 1)]$ where P is the relative pressure (ratio of the column inlet pressure P_i to the outlet pressure P_0). $P_i = P_G + P_0$ and $P = (62.9 + 754.5) / 754.5 = 1.0834$

$j = 3 / 2 (0.1737 / 0.2715) = 0.9596$

• Calculation of the carrier gas flow rate at the column temperature from the flow rate measured at the column outlet F_0 . If measurements are made with a soap-film meter it is necessary to correct the flow rate for the difference between the dry gas (column) and water saturated gas (meter) measurements. F_c is the corrected carrier gas flow rate.

$F_c = F_0 [T_c / T_a] [1 - (P_w / P_0)] = 21.78 [394.2 / 296.2] [1 - (21.068 / 754.5)] = 28.18$ ml / min.

• Column hold-up volume corresponding to the hold-up time is V_M and after correction for compressibility of the carrier gas is the corrected hold-up volume V_M° . The latter is equivalent to the gas phase volume of the column at the average column pressure and temperature. $V_M = t_M F_c = (0.23) (28.18) = 6.48$ ml and $V_M^\circ = j V_M = (0.9596) (6.48) = 6.22$ ml.

• The retention volume (V_R) is the volume of mobile phase entering the column between sample injection and the emergence of the peak maximum for the substance of interest. The corrected retention volume (V_R°) is the retention volume corrected for the compressibility of the carrier gas. $V_R = t_R F_c = (12.61) (28.18) = 355.3$ ml and $V_R^\circ = j V_R = (0.9596) (355.3) = 341$ ml.

• The adjusted retention volume (V_R') is the retention volume corresponding to the adjusted retention time and the net retention volume (V_N) is the adjusted retention volume corrected for the compressibility of the carrier gas. $V_R' = t_R' F_c = (12.61 - 0.23) (28.18) = 348.9$ ml and $V_N = j V_R' = (0.9596) (348.9) = 334.8$ ml.

• The specific retention volume (V_g°) is the net retention volume per gram of stationary phase (either liquid phase or solid adsorbent) at the column temperature. $V_g^\circ = V_N / W_L = (334.8) / (1.5115) = 221.5$ ml/g. [the specific retention volume corrected to 0°C is $V_g = V_g^\circ (273.2 / T_c)$]

that depends on the compressibility of the mobile phase. Mobile phase compressibility varies over a wide range with gases being the most compressible, liquids the least, and supercritical fluids in between. The mobile phase compressibility correction factor, j , allows the calculation of the average mobile phase velocity and solute retention volumes at the average column pressure from the experimentally measured inlet and outlet pressures in gas chromatography. The process is outlined for the example given in Table 1.2. The selection and correct use of the compressibility correction factor has generated some debate [14-19]. The gas compressibility correction factor can be specified for the column length or the solute residence time, and for ideal and non-ideal behavior of the carrier gas, all of which are different [19]. Typical usage, the conversion of volumes measured at the column outlet under ambient conditions into the corresponding volumes at the pressure averaged over the column length, assuming near ideal behavior

for the carrier gas, is illustrated in Table 1.2. In gas-solid chromatography, the situation is somewhat different: gas and analyte molecules must compete for adsorption sites on the stationary phase and distribution constants are likely to be pressure dependent reflecting the influence of different gas density gradients over the column. Corrected retention volumes, therefore, are unlikely to be invariant of the column inlet pressure. The identity of the carrier gas should also play a more significant role in establishing the relative retention order in gas-solid chromatography, as generally observed [20].

Not only variations in the pressure at constant temperature influence column-to-column retention data: the role of the column hold-up volume as well as the mass of stationary phase present in the column is also important. The net retention volume calculated from the adjusted retention volume corrects for the column hold-up volume (see Table 1.2). The specific retention volume corrects for the different amount of stationary phase present in individual columns by referencing the net retention volume to unit mass of stationary phase. Further correction to a standard temperature of 0°C is discouraged [16-19]. Such calculations to a standard temperature significantly distort the actual relationship between the retention volumes measured at different temperatures. Specific retention volumes exhibit less variability between laboratories than other absolute measures of retention. They are not sufficiently accurate for solute identification purposes, however, owing to the accumulation of multiple experimental errors in their determination. Relative retention measurements, such as the retention index scale (section 2.4.4) are generally used for this purpose. The specific retention volume is commonly used in the determination of physicochemical properties by gas chromatography (see section 1.4.2).

It is normal practice to assume that the typical carrier gases used for gas chromatography are ideal. This allows volume corrections to be made using the ideal gas laws and for gas-solute interactions to be ignored in the interpretation of retention properties. For the most exact work, it may be necessary to allow for non-ideal behavior of the gas phase by applying a correction for solute-gas phase interactions [21,22]. For carrier gases that are insoluble in the stationary phase and at moderate column inlet pressures Eq. (1.5) is a reasonable approximation

$$\ln V_N = \ln V_N(0) + 0.75 [(2B_{12} - V_1) / RT_c] [(P^4 - 1) / (P^3 - 1)] P_0 \quad (1.5)$$

where $V_N(0)$ is the net retention volume at zero column pressure drop, B_{12} the second interaction virial coefficient of the solute with the carrier gas, V_1 the solute molar volume at infinite dilution in the stationary phase (commonly replaced by the bulk molar volume), R the universal gas constant, P the relative pressure and P_0 the pressure at the column outlet (see Table 1.2 for definitions). Under normal operating conditions errors due to assuming ideality of the gas phase for simple carrier gases like hydrogen, helium and nitrogen are small, however, they increase with high solute concentrations, large column pressure drops, and low temperatures. Virial corrections are usually made only when it is desired to calculate exact thermodynamic constants from retention volume measurements. Alternatively, high-pressure gas chromatography can be used

to calculate virial coefficients. The number of accurately determined virial coefficients is small and limits the general application of Eq. (1.5).

Liquids are far less compressible than gases and for the majority of applications the influence of the column pressure drop on the retention factor in liquid chromatography is ignored. Since the column pressure drop in normal and ultrahigh pressure liquid chromatography is relatively large this practice might be questionable in some cases [23-26]. The observed retention factor when calculated from retention time is an average value reflecting the retention factor gradient over the length of the column. The observed (average) retention factor has been shown to vary linearly with the inlet pressure in a solute-specific manner. The slope is usually shallow and unless retention factors are compared at large inlet pressure differences average retention factor values are nearly constant. However, it is now clear that retention factors are not invariant of pressure over the full range of inlet pressures used in modern liquid chromatography, and this has some implications for determining physicochemical properties by liquid chromatography but is less important for analysis.

Fluids are highly compressible and density gradients along the column associated with the column pressure drop result in significant retention factor changes as a function of local density. These changes are complex and usually modeled by empirical relationships (section 7.5).

1.4.2 Property Estimations

Gas chromatography is widely used to determine solution and adsorption thermodynamic properties [21,22,27-32]. Compared to classical static methods it has several advantages. Measurements can be made for impure samples, very small sample sizes are sufficient, and easy variation of temperature is provided. For the most exact measurements precise flow, pressure, and temperature control is needed that may require modification to a standard analytical gas chromatograph. The free energy, enthalpy, and entropy of mixing or solution, and infinite dilution solute activity coefficients can be determined from retention measurements made at infinite dilution (Henry's law region) in which the value of the activity coefficient (also the gas-liquid distribution constant) can be assumed to have a constant value. At infinite dilution the solute molecules are not sufficiently close to exert any mutual attractions, and the environment of each may be considered to consist entirely of solvent molecules. The activity coefficient and the specific retention volume are related by $V_g = (273.2 R) / (M_2 \gamma_1 P_1^\circ)$ where M_2 is the molecular weight of the solvent, γ_1 the solute activity coefficient at infinite dilution, and P_1° the saturation vapor pressure of the pure solute at the given temperature. Ideally, activity coefficients calculated from the above relationship should be corrected for fugacity (solute-solute interactions), imperfect gas behavior, and interfacial adsorption. The first two corrections may introduce errors of ca. 1-5% in the value of the activity coefficient depending on the circumstances of the measurement; ignoring the importance of interfacial adsorption as a retention mechanism may make values for the activity coefficient completely meaningless (section 2.4.1). Typical infinite dilution activity coefficients for nonionic solvents, used in gas chromatography, have values in the range

0.3 to 50 [29,31]. Positive deviations from Raoult's law ($\gamma_1 > 1$) are common for the high-molecular-weight solvents generally used in gas chromatography. Activity coefficients much less than one indicate strong solute-stationary phase interactions.

The gas-liquid distribution constant (K_L), moles of solute per unit volume of liquid / moles of solute per unit volume of gas phase, is evaluated from the specific retention volume using the relationship $V_g = (273.2 K_L) / (T_c \rho_c)$ where ρ_c is the liquid phase density at the column temperature [32]. Alternatively, extrapolation of the net retention volume measured at several different phase loadings to an infinite stationary phase volume allows the gas-liquid distribution constant to be obtained independent of accompanying contributions from interfacial adsorption (section 2.4.1). The gas-liquid distribution constant can then be used to calculate values of the specific retention volume that are corrected for contributions to retention arising from interfacial adsorption. Also the partial molar Gibbs free energy of solution for a solute at infinite dilution (ΔG°) in the stationary phase can be obtained directly from the gas-liquid distribution constant using $\Delta G^\circ = -RT_c \ln K_L$. From the slope of a plot of $\log V_g$ against the reciprocal of the column temperature over a narrow temperature range, 10-30 K, the enthalpy of solution is obtained. The entropy for the same process is obtained from a single value of the specific retention volume and the value of the enthalpy of solution calculated as just described [34-36].

Compared to gas chromatography liquid chromatography is used far less for physicochemical measurements [37,38]. Inadequate knowledge of the true composition of the stationary phase and the absence of quantitative models for the accurate description of retention are the principal reasons for this. A few exceptions are the determination of equilibrium constants that affect the form of a solute in the mobile phase (ion dissociation, complexation, confirmation, etc.) Also, indirect property determinations based on quantitative structure – activity relationships (QSAR) and quantitative structure – property relationships (QSPR) [39-43]. QSAR and QSPR relationships are based on the identification of an empirical correlation between a retention property in a chromatographic system, usually the retention factor and another (usually) equilibrium property of a chemical or biological system. Typical examples include the octanol-water distribution constant, the distribution of compounds across biological membranes, aquatic toxicity of organic compounds, the soil-water distribution constant, etc. These relationships are often, although not exclusively, of the form $\log P = a \log k + b$ where P is some equilibrium dependent property and a and b are empirical regression constants. Once the correlation equation is established using known values of $\log P$ further values of $\log P$ can be estimated from the correlation equation by measuring their chromatographic retention. This provides an inexpensive and rapid method for estimating properties that are difficult and expensive to determine by direct measurement.

1.4.3 Linear Free Energy Relationships

The free energy of transfer of a solute between two phases can be described as the linear sum of contributing processes delineated by a suitable model. For chromatographic

and liquid-liquid distribution systems a cavity model provides a general approach for characterizing the contribution of solvent-solvent and solvent-solute interactions to equilibrium properties [44,45]. Firstly, a cavity of a suitable size to accommodate the solute is constructed in the solvent, with the solvent molecules in the same state as in the bulk solvent. The energy required for this process depends on the forces holding the solvent molecules together, and the solute's size. Cavity formation requires work and opposes solute transfer. In the second step the solvent molecules are reorganized into their equilibrium position round the solute. The free energy for this process is approximately zero and can be neglected. Although it should be pointed out, however, that the enthalpy and entropy of reorganization may be considerable – the free energy is effectively zero because of compensation, as in the melting of ice at 0°C. Finally, the solute is inserted into the cavity and various solute-solvent interactions are set up. For nonionic solutes these are identified as dispersion, induction, orientation, and hydrogen bonding. If two condensed phases are involved in the equilibrium then the free energy of transfer is equivalent to the difference in cavity formation and solute-solvent interactions in the two phases. For transfer from an ideal gas phase to a solvent at infinite dilution the free energy of transfer is equal to the difference in free energy of cavity formation in the solvent and the strength of solute-solvent interactions.

To move from a qualitative to a quantitative picture the individual free energy contributions to the solvation process identified above must be delineated in a quantitative form. Within the framework of a linear free energy relationship the contributions of individual intermolecular interactions are represented as the sum of product terms made up of solute factors (descriptors) and complementary solvent factors (system constants). Thus a solute has a certain capability for a defined intermolecular interaction and its contribution to the solution free energy is the product of the capability of the solute and solvent for that interaction. Kamlet, Taft and their co-workers [44,46] developed one of the earliest general approaches to the quantitative characterization of solute-solvent interactions based on solvatochromism. Solvatochromic parameters were defined by the influence of environment (solvent effects) on the absorption spectra of select compounds and normalized to provide roughly equivalent scales. This method has been widely used to determine the dipolarity/polarizability (π^*), the hydrogen-bond acidity (α) and hydrogen-bond basicity (β) of common solvents [47]. Kamlet, Taft and their co-workers extended their solvatochromic parameters to solute effects, assuming that the solvent parameters could be taken as an estimate of solute properties. This is at best a rough approximation. In a bulk solvent, each molecule is surrounded by molecules like itself, while as a solute it is surrounded by solvent molecules that are different to it. Compounds, such as alcohols, that are highly associated as solvents are expected to behave differently as monomeric solute molecules. However, there are also fundamental limitations to this approach. The solvatochromic parameters are related to spectroscopic energy differences, that is the influence of solvent effects on the ground and excited states of the selected indicator compounds, which are not free energy processes *per se*. Secondly, although some parameter estimate rules have been developed, there is no protocol for the determination of the Kamlet-Taft parameters for

additional (especially solid) compounds. In order to construct a correlation equation that has a sound physical interpretation, it is necessary that the various descriptors should be related to Gibbs free energy. Descriptors meeting this requirement were developed by Abraham and co-workers [45,48-51] and are to be preferred to the solvatochromic parameters for chromatographic retention studies and for wider application to solute properties that can be characterized by a distribution constant. Before describing Abraham's solvation parameter model, it is necessary to reiterate that the solute descriptors for the solvation parameter and solvatochromic models are not the same, although they are often mistaken or misused as such in the contemporary literature. The solvation parameter model is also unrelated to the solubility parameter model.

The solvation parameter model for distribution between two condensed phases, Eq. (1.6) or (1.6a), and transfer from the gas phase to a solvent, Eq. (1.7) or (1.7a), are set out below in the form generally used in chromatography.

$$\log SP = c + mV_X + rR_2 + s\pi_2^H + a\sum\alpha_2^H + b\sum\beta_2^H \quad (1.6)$$

$$\log SP = c + vV + eE + sS + aA + bB \quad (1.6a)$$

$$\log SP = c + rR_2 + s\pi_2^H + a\sum\alpha_2^H + b\sum\beta_2^H + l\log L^{16} \quad (1.7)$$

$$\log SP = c + eE + sS + aA + bB + lL \quad (1.7a)$$

Eqs. (1.6) and (1.6a) and (1.7) and (1.7a) are identical but written with different symbols. Eqs. (1.6) and (1.7) have been commonly used in the literature following Abraham's description of the solvation parameter model. Recently, Abraham has suggested replacement of these equations with (1.6a) and (1.7a) to simplify representation of the model [52,53]. It is likely that Eqs. (1.6a) and (1.7a) will replace Eqs. (1.6) and (1.7) as the general representation of the solvation parameter model in the future.

SP is some free energy related solute property such as a distribution constant, retention factor, specific retention volume, relative adjusted retention time, or retention index value. Although when retention index values are used the system constants (lowercase letters in italics) will be different from models obtained with the other dependent variables. Retention index values, therefore, should not be used to determine system properties but can be used to estimate descriptor values. The remainder of the equations is made up of product terms called system constants (r, s, a, b, l, m) and solute descriptors ($R_2, \pi_2^H, \sum\alpha_2^H, \sum\beta_2^H, \log L^{16}, V_X$). Each product term represents a contribution from a defined intermolecular interaction to the solute property. The contribution from cavity formation and dispersion interactions are strongly correlated with solute size and cannot be separated if a volume term, such as the characteristic volume [V_X in Eq. (1.6) or V in Eq. (1.6a)] is used as a descriptor. The transfer of a solute between two condensed phases will occur with little change in the contribution from dispersion interactions and the absence of a specific term in Eq. (1.6) to represent dispersion interactions is not a serious problem. For transfer of a solute from the gas phase to a condensed phase this

Table 1.3

Calculation of solute descriptor values for the solvation parameter model

- Calculation of McGowan's characteristic volume, V_X (or V), for toluene

Atomic volumes: C = 16.35, H = 8.71, N = 14.39, O = 12.43, F = 10.48, Si = 26.83, P = 24.87, S = 22.91, Cl = 20.95, Br = 18.23, I = 34.53. Subtract 6.56 for each bond of any type.

Toluene = 7 carbon atoms + 8 hydrogen atoms – 15 bonds = $114.45 + 69.68 - 98.40 = 85.73$ in $\text{cm}^3 \cdot \text{mol}^{-1}$.

After scaling $V_X = 0.857$ in $\text{cm}^3 \cdot \text{mol}^{-1}/100$.

- Calculation of the excess molar refraction, R_2 (or E), for toluene using Eq. (1.8). The refractive index for toluene (n) at 20°C (sodium D-line) = 1.496

$R_2 = 8.57 (0.292) + 0.5255 - 2.832 (0.857) = 0.601$ in $\text{cm}^3 \cdot \text{mol}^{-1}/10$.

- Estimation of solute descriptors for 2,6-dimethoxyphenol from liquid-liquid distribution constants. V_X and R_2 were calculated as above giving 1.1743 and 0.840, respectively. Other solute descriptors were obtained as the best-fit values from the distribution systems given below

Distribution system	log K(calc.)	log K(exp.)	Best-fit values		
Water-octanol	1.10	1.15	π_2^H	$\Sigma \alpha_2^H$	$\Sigma \beta_2^H$
Water-ether	0.79	0.74	1.41	0.13	0.71
Water-olive oil	0.56	0.57			
Water-hexadecane	-0.35	-0.36			
Water-cyclohexane	-0.15	-0.15			

is no longer the case and the solvation equation must be set up to account for the contribution of dispersion interactions to the free energy of solute transfer. Abraham handled this problem by defining a second descriptor for the contribution of cavity formation and dispersion interactions [$\log L^{16}$ in Eq. (1.7) or L in Eq. (1.7a)]. This term includes not only solute-solvent dispersion interactions, but also the cavity effect making the V_X term in Eq. (1.6) redundant. For general applications Eq. (1.6) is the form of the model suitable for characterizing chromatographic retention in systems with two condensed phases, such as liquid and micellar electrokinetic chromatography. Eq. (1.7) is suitable for characterizing retention in gas chromatography, and more generally in two phase systems where one component is a gas.

The solute descriptors used in Eq. (1.6) and (1.7) must be free energy related properties to correlate with chromatographic retention. It is also important that the solute descriptors are accessible for a wide range of compounds by either calculation or simple experimental techniques, otherwise the models would lack practical utility. McGowan's characteristic volume, V_X or V in units of $\text{cm}^3 \cdot \text{mol}^{-1} / 100$, can be calculated for any molecule whose structure is known by simple summation rules, Table 1.3 [49,54]. Each atom has a defined characteristic volume and the molecular volume is the sum of all atomic volumes less $6.56 \text{ cm}^3 \cdot \text{mol}^{-1}$ for each bond, no matter whether single, double or triple. For complex molecules the number of bonds, B , is easily calculated from the algorithm $B = N - 1 + R$ where N is the total number of atoms, and R is the number of rings. $\log L^{16}$ or L is the solute gas-liquid distribution constant (also referred to as the Ostwald solubility coefficient) on hexadecane at 298 K. For volatile solutes it can be determined directly [50]. For all compounds of low volatility, it is determined by back calculation from gas chromatographic retention measurements

on nonpolar stationary phases at any convenient temperature [56-60]. Suitable stationary phases are those for which the system constants $a \approx b \approx s \approx 0$ in Eq. (1.7).

The solute excess molar refraction, R_2 or E , models polarizability contributions from n - and π -electrons. The solute molar refraction is too closely related to solute size to be used in the same correlation equation as V_X . To avoid correlation between the molar refraction and V_X , an excess molar refraction, R_2 , was defined as the molar refraction for the given solute, less the molar refraction for an n -alkane of the same characteristic volume [61,62]. The excess molar refraction is simply calculated from the refractive index of the solute at 20°C for the sodium D-line, η , as indicated by Eq. (1.8)

$$R_2 = 10V_X[(\eta^2 - 1) / (\eta^2 + 2)] - 2.832 V_X + 0.526 \quad (1.8)$$

The units used for V_X in Eq. (1.8) are $\text{cm}^3 \cdot \text{mol}^{-1} / 100$, and therefore R_2 is given in $\text{cm}^3 \cdot \text{mol}^{-1} / 10$. The use of Eq. (1.8) to calculate the excess molar refraction is straightforward for liquids but even for solids refractive index values are easily estimated using available software for molecular property estimations. In addition, R_2 , like the molar refraction, is almost an additive quantity, and values for solids can be estimated through addition of fragments with known R_2 values [45,63-65].

In developing the solvation parameter model Abraham and coworkers commenced the process by defining descriptors for solute hydrogen-bond acidity (α_2^H) and solute hydrogen-bond basicity (β_2^H). The superscript (H) indicates the origin of the scale and the subscript (2) that the descriptors are solute properties. Initially these solute descriptors were determined from 1:1 complexation constants measured in an inert solvent [66,67]. These studies also led to scales that had a zero origin. A problem still remained, however, when these descriptors were used to characterize distribution processes. The influence of solute structure on the distribution process will be a consequence of hydrogen bonding of the solute to any surrounding solvent molecules, not just to one. What are needed are scales of "summation" or "overall" hydrogen bonding that refer to the propensity of a solute to interact with a large excess of solvent molecules. These hydrogen-bond descriptors are denoted as $\sum \alpha_2^H$ and $\sum \beta_2^H$ to distinguish them from the 1:1 descriptors. New values of the effective hydrogen bonding solute descriptors are now determined in conjunction with other solute descriptors using liquid-liquid distribution and chromatographic measurements [49,68,69]. A minor complication is that certain solutes (sulfoxides, anilines, pyridines) show variable hydrogen-bond basicity in distribution systems where the organic phase absorbs appreciable amounts of water [68]. A new solute descriptor $\Sigma \beta_2^0$ was defined for these solutes and should be used in octanol-water distribution systems, for example, and for reversed-phase and micellar electrokinetic chromatography. For the same solutes $\sum \beta_2^H$ should be used for all other applications and always for gas chromatography. Except for the solute types indicated above, the two hydrogen-bond basicity scales are identical. It should also be noted that the scales of hydrogen-bond acidity and basicity are generally unrelated to proton transfer acidity and basicity expressed by the pK_a scale.

It would be useful to have descriptors that were related to the propensity of a solute to engage in dipole-dipole and induced dipole-dipole interactions. In the event, it proved

impossible to separate out descriptors for the two types of interactions, and Abraham and coworkers [61] constructed a solute descriptor for dipolarity/polarizability, π_2^H or S , combining the two interactions. The dipolarity/polarizability descriptor was initially determined through gas chromatographic measurements on polar stationary phases [56,57,70], but is now more commonly determined in combination with the hydrogen-bonding solute descriptors from liquid-liquid distribution constants and chromatographic measurements [45,50,69].

Solute descriptors are available for about 4,000 compounds, with some large compilations reported [42,43,49,58,68]. For additional compounds estimation methods are available using fragment constants [46,50,63-65]. An early version of a software program to estimate solute descriptors from structure has appeared [71]. In all other cases it is possible to calculate V_X and R_2 and determine the other descriptors from experimental distribution constants and chromatographic measurements. If data is available for a particular solute in three systems with significantly different system constants then π_2^H , $\Sigma\alpha_2^H$, $\Sigma\beta_2^H$ can be determined as the solution to three simultaneous equations. If the number of equations is larger than the number of descriptors to be determined, the descriptor values that give the best-fit solution (i.e., the smallest standard deviation in the observed and calculated log SP values) are taken [69].

The system constants in Eqs. (1.6) and (1.7) are obtained by multiple linear regression analysis for a number of solute property determinations for solutes with known descriptors. The solutes used should be sufficient in number and variety to establish the statistical and chemical validity of the model [72-74]. In particular, there should be an absence of significant cross-correlation among the descriptors, clustering of either descriptor or dependent variable values should be avoided, and an exhaustive fit should be obtained. Table 1.4 illustrates part of a typical output. The overall correlation coefficient, standard error in the estimate, Fischer F-statistic, and the standard deviation in the individual system constants are used to judge whether the results are statistically sound. An exhaustive fit is obtained when small groups of solutes selected at random can be deleted from the model with minimal change in the system constants.

The system constants are more than regression constants and contain important chemical information about the system. Consequently, not only must the system constants be statistically sound but they must make chemical sense as well. The system constants reflect the difference in solute interactions in the two phases, except for gas chromatography, where the system constants reflect stationary phase properties alone. The r (or e) system constant indicates the tendency of the phases to interact with solutes through π - and n -electron pairs; the s system constant for the tendency of the phases to interact with solutes through dipole-type interactions; the a system constant denotes the difference in hydrogen-bond basicity between phases (because acidic solutes will interact with a basic phase); and the b system constant is a measure of the difference in hydrogen-bond acidity of the phases (because basic solutes will interact with an acidic phase). The l system constant is a measure of the energy required for cavity formation and the strength of dispersion interactions in gas chromatography. The m (or v) system constant is a measure of the difference in cavity formation in the two condensed phases

Table 1.4

An example of part of the output for fitting the solvation parameter model to a reversed-phase chromatographic system by multiple linear regression analysis

Solute	Descriptors					log k	
	V_X	R_2	π_2^H	$\Sigma\alpha_2^H$	$\Sigma\beta_2^0$	Experimental	Predicted
Phenol	0.775	0.805	0.89	0.60	0.31	-0.306	-0.273
Benzyl Alcohol	0.916	0.803	0.87	0.33	0.56	-0.268	-0.252
Aniline	0.816	0.955	0.96	0.26	0.50	-0.386	-0.380
Toluene	0.857	0.601	0.52	0	0.14	0.553	0.524
Ethylbenzene	0.998	0.613	0.51	0	0.15	0.997	0.937
Naphthalene	1.085	1.340	0.92	0	0.20	1.185	1.256
Benzaldehyde	0.873	0.820	1.00	0	0.39	-0.017	-0.011
Nitrobenzene	0.890	0.871	1.11	0	0.28	0.143	0.225
Chlorobenzene	0.838	0.718	0.65	0	0.07	0.618	0.597
Acetophenone	1.014	0.818	1.00	0	0.49	0.275	0.236

<u>Cross-correlation matrix (r^2)</u>					<u>Model Statistics</u>	<u>System constants</u>
V_X	1.00					$m = 2.99 (0.07)$
R_2	0.19	1.00			$R = 0.994$	$r = 0.46 (0.05)$
π_2^H	0.00	0.33	1.00		$SE = 0.07$	$s = -0.44 (0.05)$
$\Sigma\alpha_2^H$	0.29	0.22	0.03	1.00	$F = 570$	$a = -0.30 (0.05)$
$\Sigma\beta_2^0$	0.00	0.03	0.42	0.17	$n = 40$	$b = -1.88 (0.08)$
						$c = -1.82 (0.07)$

together with any residual dispersion interactions that are not self-canceling. For the reversed-phase chromatographic system summarized in Table 1.4 the factors leading to retention (increase in log k) are the favorable cohesion properties of the stationary phase and electron lone pair interactions (m and r system constants have a positive sign). All polar interactions favor solubility in the mobile phase (s , a , and b system constants have a negative sign) and result in reduced retention. From the relative magnitude of the system constants the most important solute property resulting in increased retention is its size and the most important factor reducing retention is its hydrogen-bond basicity. These are general properties of reversed-phase chromatographic systems. Applications of the solvation parameter model for material characterization, retention prediction, and method development in gas chromatography (section 2.3.5), reversed-phase liquid chromatography (section 4.3.1.3), thin-layer chromatography (section 6.7.2), micellar electrokinetic chromatography (section 8.3.3) and solid-phase extraction [75,76] are discussed elsewhere.

1.4.4 Exothermodynamic Relationships

A number of general relationships are used in chromatography to relate retention to solute or experimental variables. Apart from the influence of temperature these are not formal thermodynamic relationships but nevertheless are very useful for predicting retention properties and establishing retention mechanisms. They can be formulated as linear free energy relationships on the assumption that the free energy of solute

transfer from the mobile to the stationary phase is an additive property. In which case the free energy of transfer can be taken as the sum of the free energy for each structural element of the solute. By way of example, for compounds belonging to a homologous series, such as $\text{CH}_3(\text{CH}_2)_n\text{OH}$, the total free energy of transfer can be decomposed into $\Delta G^\circ(\text{CH}_3) + n\Delta G^\circ(\text{CH}_2) + \Delta G^\circ(\text{OH})$. If we further assume that the difference between the methylene group and methyl group can be ignored then linear plots are expected for a plot of the logarithm of the retention property against carbon number (n or $n + 1$ in this case). This is generally what is observed in gas chromatography at a constant temperature and liquid chromatography at a constant mobile phase composition when $n \geq 3$ [77-80]. The logarithm of the chromatographic retention property can be any of the following; adjusted retention time, adjusted retention volume, specific retention volume, retention factor, or distribution constant. In the case of gas-liquid chromatography the saturation vapor pressure, boiling point or refractive index can be substituted for the carbon number, since these properties are often highly correlated with the carbon number for members of a homologous series. These relationships are sometimes referred to as Martin's rule, after the pioneer of partition chromatography (A. J. P. Martin) who anticipated and demonstrated their existence. From this concept Martin also explained the remarkable power of chromatography to separate substances that differ only slightly in their structure. For two compounds that differ in a structural element the difference in free energy of transfer (separation) is proportional to the corresponding free energy change for that structural element but not for the rest of the molecule. This explains why proteins that differ only in a single amino acid can be separated. As an extension of this idea functional group contributions for a large number of substituent groups on a range of parent structures have been derived to predict retention of simple substituted compounds from parent structures in reversed-phase liquid chromatography [77,78] and gas-liquid chromatography [40]. For polyfunctional compounds this approach has met with limited success because the additivity principle fails to account for intramolecular interactions between substituent groups and steric factors which influence solute-solvent interactions in a solute characteristic manner.

A similar linear logarithmic relationship, known as a van't Hoff plot, usually exists between adjusted retention data and the reciprocal of column temperature in gas, liquid (constant composition) and supercritical fluid (constant density) chromatography. The effect of temperature on retention is based on the Gibbs-Helmholtz equation and has a sound thermodynamic basis, Eq. (1.9)

$$\ln k = -(\Delta H^\circ / RT) + (\Delta S^\circ / R) + \ln \beta \quad (1.9)$$

where ΔH° is the standard enthalpy and ΔS° the standard entropy of transfer of the solute from the mobile to stationary phase, R the gas constant, T temperature (K) and β the phase ratio. The plot of $\ln k$ against $1/T$ will be linear provided the change in heat capacity for the transfer is zero (i.e., ΔH° , ΔS° and β are temperature invariant). Nonlinear plots with sharp discontinuities in their slopes are observed for mixed retention mechanisms or change in solute or stationary phase conformation

that influences sorption [79,81,82]. Linear van't Hoff plots can be used to determine enthalpies (from the slope) and entropies of transfer (from the intercept) although the latter can be difficult to determine owing to the non-trivial nature of calculating the phase ratio, particularly for liquid and supercritical fluid chromatography.

The influence of temperature on resolution is not as straightforward to predict as that of retention. Changing temperature affects thermodynamic properties (retention) and kinetic properties (peak shapes) at the same time [81-83]. Higher temperatures in gas chromatography decrease the cohesive properties and capacity of stationary phases for polar interactions resulting in a characteristic solute dependent reduction in retention [50,84,85]. For mixtures with a narrow range of retention properties there is usually an optimum temperature at which peak separations are maximized. In practice higher temperatures than this may be preferred if an adequate peak separation is maintained, more favorable peak shapes result, and faster separations are obtained. This is because temperature also influences diffusion coefficients in the mobile and stationary phases and the viscosity of the carrier gas. In general, the operating pressure will not be the limiting factor in gas chromatography and higher inlet pressures can be used to maintain an optimum mobile phase velocity to offset the increase in the carrier gas viscosity at higher temperatures. Higher temperatures, as well, result in improved mass transfer properties and narrower peaks counteracting any decrease in peak spacing. The net result is that higher temperatures can result in changes in resolution that can be for the better or worse.

The vast majority of liquid chromatographic separations are carried out at ambient temperature for convenience and because ambient temperature provides reasonable column efficiency for low molecular weight solutes. Elevated temperatures reduce the viscosity of the mobile phase and enhance solute diffusion [81,86,87]. This is expected to result in lower operating pressures, improved mass transfer properties but increased longitudinal diffusion (these properties are discussed in section 1.5.2). In terms of peak shapes this can be advantageous or disadvantageous depending on the mobile phase velocity and whether longitudinal diffusion or mass transfer properties dominate. Higher temperatures will also change the column selectivity so that predicting changes in resolution as a function of temperature can be difficult. The simultaneous optimization of temperature and mobile phase composition is considered desirable for method development in liquid chromatography, although the possibility of exploiting temperature as an optimization variable is often ignored [87-89]. Temperature variation and composition variation show similar trends in retention, but within the easily accessible range for both variables the capacity to change retention is much greater for composition variation [89,90]. The predominant influence of higher temperatures in reversed-phase liquid chromatography is to decrease retention by a reduction in the difference in cohesive energy between the mobile and stationary phases and to decrease the hydrogen-bond acidity of the mobile phase relative to the stationary phase, Figure 1.3 [89]. Changes in other polar interactions are less significant. Temperature variation in reversed-phase liquid chromatography, therefore, will have the largest effect on peak spacing of compounds that differ in size and hydrogen-bond basicity. The

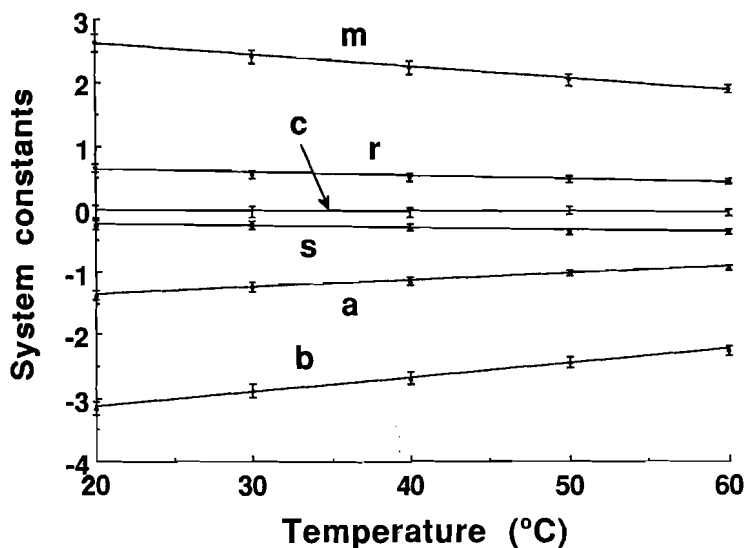


Figure 1.3. Variation of the system constants of the solvation parameter model (section 1.4.3) with temperature for 37 % (v/v) propan-2-ol in water on the porous polymer PLRP-S stationary phase. The m constant reflects the difference in cohesion and dispersive interactions, r constant lone-pair electron interactions, s constant dipole-type interactions, a constant hydrogen-bond basicity and b constant hydrogen-bond acidity between the mobile and stationary phases. (From ref. [89]; ©The Royal Society of Chemistry).

use of very high temperatures in liquid chromatography is quite recent, for example, pressurized water up to 200°C [90], the separation of proteins on nonporous sorbents at up to 120°C [91], and the separation of varied compounds on polymer encapsulated sorbents at up to 200°C [92]. Here the main interest has been to exploit pure water as a mobile phase or to obtain fast separations by increasing the separation efficiency at high flow rates. These extreme temperatures are manageable from an instrumental perspective but careful stationary phase selection is important because many chemically bonded phases degrade rapidly at high temperatures. Subambient temperatures are generally reserved for the separation of labile solutes where the inefficient column operation, long separation times, and high column pressure drops are justified by the need to maximize the peak separation of enantiomers or protect the solute from decomposition, denaturation, or conformational changes [83,93,94].

Plots of the standard entropy, or better standard free energy, against the standard enthalpy referred to as enthalpy-entropy compensation plots, are a useful tool for establishing the similarity of the retention mechanism for different solutes [25,79,83,95,96]. When enthalpy-entropy compensation occurs the plots are linear and the slope is called the compensation temperature. All compounds have the same retention at the compensation temperature, although their temperature dependence may differ. This is most likely to occur for processes governed by a single retention mechanism. All related processes

that have the same compensation temperature proceed via the same mechanism. Some care is needed in the interpretation of these plots though. It is entirely possible to observe adventitious correlations between the standard enthalpy and entropy from spurious statistical effects that arise from the least squares fitting of the data [95-97]. This can happen when the entropy and enthalpy are both obtained from the same van't Hoff plot. When the enthalpy-entropy compensation is real the plot of the retention factor ($\ln k$) against the reciprocal of temperature for all solutes must show a single intersection point. In addition the enthalpy-entropy compensation can be considered real only when the analysis of variance (ANOVA) indicates that the variance explained by the plot exceeds the random experimental variance according to an appropriate F-test. When a highly variegated set of solutes is chosen and enthalpy-entropy compensation is observed then one can be reasonably certain the retention process is dominated by a single factor. Enthalpy-entropy compensation is invariably observed for members of a homologous series since these have virtually an identical capacity for polar interactions and differ only by size. Besides temperature, enthalpy-entropy compensation can be observed for other system parameters. For example, organic modifier concentration in reversed-phase liquid chromatography, molal salt concentration in hydrophobic interaction chromatography, or a stationary phase property such as the carbon number of different bonded alkyl chains [79].

1.4.5 General Elution Problem

Separations employing constant temperature in gas chromatography, constant mobile phase composition in liquid chromatography, and constant density in supercritical fluid chromatography fail to provide useful results for samples containing components with a wide range of distribution constants. In gas chromatography, for example, there is an approximate exponential relationship between retention time and solute boiling point at a constant (isothermal) column temperature. Consequently, it is impossible to establish a suitable compromise temperature for the separation of mixtures with a boiling point range exceeding about 100°C. In liquid and supercritical fluid chromatography similar problems arise when the affinity of the solute for the stationary phase is sufficient to preclude convenient elution at a constant mobile phase composition or density, respectively. This is generically referred to as "the general elution problem" and is characterized by long separation times, poor separations of early eluting peaks and poor detectability of late eluting peaks due to band broadening. The general solution to this problem is the use of programmed separation modes. For gas chromatography the useful program modes are temperature and flow programming (section 2.4.3); for liquid chromatography mobile phase composition, flow and temperature programming (section 4.4.6); and for supercritical fluid chromatography density, composition and temperature programming (section 7.5.1). The programmed modes provide a complete separation of mixtures with a wide range of retention properties in a reasonable time. Neither constant nor programmed modes are superior to one another. They are complementary with the properties of the sample deciding which approach is adopted.

Temperature programming is the most popular programmed separation mode in gas chromatography. Stationary phases of high thermal stability allow wide temperature ranges to be used and temperature is easily adjusted and controlled using the forced air circulation ovens in general use with modern instruments. Flow programming is easily achieved with instruments fitted with electronic pressure control but is limited by the narrow pressure range usually available. It can be used to separate thermally labile compounds at a lower temperature than required for temperature programmed separations. On the other hand, flow programming results in a loss of efficiency for late eluting peaks and presents difficulties in calibrating flow sensitive detectors. Composition programming is the common programmed separation mode in liquid chromatography. Temperature and flow programming are not generally used except in special circumstances. The high flow resistance of packed columns and limited operating pressures results in a narrow range of flow rates and therefore retention variation compared with composition variation. In addition, volumetric dilution of later eluting peaks reduces sample detectability with concentration-sensitive detectors. Radial temperature gradients in normal bore columns render temperature programming impractical, causing a loss of efficiency and the formation of asymmetric peaks [98]. The smaller radius and reduced mass of microcolumns makes them more amenable to temperature programming techniques where this method of reducing separation times becomes more interesting on account of the greater difficulty of constructing gradient forming devices with very low mixing volumes [98-102]. In supercritical fluid chromatography density and composition programmed modes are both popular. When a single fluid is used as mobile phase then density programming is used. For fluids mixed with an organic solvent composition programming is more common although density programming may still be used, particularly at low solvent compositions. Simultaneous temperature programs with density and composition programs are sometimes applied to improve band spacing in the separation.

1.5 BAND BROADENING

If the sample is introduced as a sharp rectangular pulse into a column the individual separated sample components when they leave the column are broadened about their characteristic retention value in proportion to the time each component remained in the column. This characteristic change in the appearance of bands in the chromatogram results from kinetic factors referred to in total as band broadening. Zone is sometimes used for band, and dispersion or spreading for broadening, resulting in a number of names for the same process. For consistency we will call the process band broadening. The extent of band broadening determines the chromatographic efficiency, conventionally expressed as either the number of theoretical plates or simply the plate number (N), or the height equivalent to a theoretical plate (HETP) or simply plate height (H). If the column is assumed to function as a Gaussian operator then the column efficiency is readily expressed in terms of the peak retention time (t_R) and

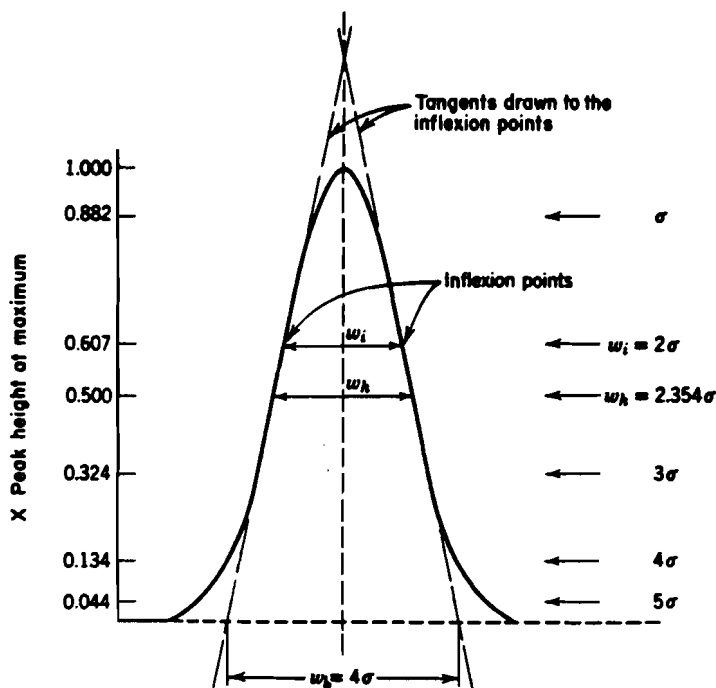


Figure 1.4. Characteristic properties of a Gaussian peak.

band variance in time units (σ_t) according to $N = (t_R / \sigma_t)^2$. In practice, various peak width measurements are frequently used based on the properties of a Gaussian peak, Figure 1.4, and Eq. (1.10)

$$N = a(t_R / w)^2 \quad (1.10)$$

where w_i is the peak width at the inflection point when $a = 4$, w_h the peak width at half height when $a = 5.54$, and w_b the peak width at the base when $a = 16$. Alternatively the ratio of the peak height to the area of a Gaussian peak can be used to define N

$$N = 2\pi(t_R h / A)^2 \quad (1.11)$$

where h is the peak height and A the peak area. The plate height, H , is given by the ratio of the column length (L) to the column plate count by $H = L / N$. Column efficiency can also be measured as the effective plate number (N_{eff}) by substituting the adjusted retention time, t_R' for the retention time in Eqs. (1.9) and (1.10). The effective plate number is considered more fundamentally significant than the plate number since it measures only the band broadening that occurs during the time the solute interacts with the stationary phase. The two measures of column efficiency are related by $N_{\text{eff}} = N [k /$

$(1 + k)^2$ where k is the retention factor. For a weakly retained solute, for example $k = 1$, N_{eff} will be only 25% of the value of N ; however, for well-retained solutes, $k > 10$, N_{eff} and N will be approximately the same. For useful column comparisons N_{eff} and N should be determined for well-retained solutes; at low k values N will be speciously high and misrepresent the actual separation performance that can be obtained from a particular column in normal use. Also for comparative purposes, it is general practice to normalize the value of N_{eff} and N on a per meter of column length basis. For many of the relationships discussed in this chapter, N and N_{eff} can be used interchangeably.

The terms plate number and plate height have their origin in the plate model of the chromatographic process [103-108]. This model, originally proposed by Martin and Synge in 1941 [109], was an extension of distillation and countercurrent liquid-liquid distribution theory to the new technique of partition chromatography. It is only of historic interest now, having been replaced by more realistic rate theory models. The plate model, however, has contributed significantly to the terms used to describe band broadening in chromatography and is briefly discussed for that reason. The plate model assumes that the column can be visualized as being divided into a number of volume elements or imaginary sections called plates. At each plate the partitioning of the solute between the mobile and stationary phase is rapid and equilibrium is reached before the solute moves onto the next plate. The solute distribution constant is the same in all plates and is independent of the solute concentration. The mobile phase flow is assumed to occur in a discontinuous manner between plates and diffusion of the solute in the axial direction is negligible (or confined to the volume element of the plate occupied by the solute). The main problem with the plate model is that it bears little physical resemblance to the chromatographic process. Axial diffusion is a significant source of band broadening in chromatography, the distribution constant is independent of concentration only over a narrow concentration range, and, quite obviously, the assumption that flow occurs in a discontinuous manner is false. In the practical sense its largest shortcoming is that it fails to relate the band broadening process to the experimental parameters (e.g., particle size, mobile phase velocity, etc.) that are open to manipulation by the investigator. Nevertheless, the measured quantities N and H are useful parameters for characterizing chromatographic efficiency and are not limited by any of the deficiencies in the plate model itself. More realistic models developed from rate theory enable a similar expression for the plate number to be derived. The general connection is that all these models result in a Gaussian-like distribution for the band broadening process, but from rate theory we can relate band broadening to the experimental variables, and in that way establish an experimental basis for kinetic optimization of the chromatographic process.

1.5.1 Flow Through Porous Media

For an understanding of band broadening in chromatographic systems, the linear velocity of the mobile phase is more important than the column volumetric flow rate. Complications in identifying a suitable velocity arise for compressible mobile phases

with pressure-driven systems. In this case the local velocity at any position in the column will depend on the flow resistance of the column and the ratio of the column inlet to outlet pressure. An average linear velocity, u , is always available as the ratio of the column length to the retention time of an unretained solute (L / t_M). For open tubular columns this definition is unambiguous. For packed columns the measured value will depend on the ability of the unretained solute to probe the pore volume of the stationary phase. Two extreme values are possible for an unretained solute with total access to the pore volume and an unretained solute that is fully excluded from the pore volume. For porous stationary phases the mobile phase trapped within the pores is generally stagnant and for kinetic optimization the mobile phase velocity through the interparticle volume, the interparticle velocity, u_e , is probably more fundamentally significant [110,111].

Since many chromatographic experiments are performed with the column exit at ambient conditions, and many solute properties are known for these conditions, the experimentally accessible outlet velocity, u_o , can be useful. The mobile phase velocity and flow rate in an open tubular column are simply related by $u_o = F_c / A_c$ where F_c is the fully corrected column volumetric flow rate and A_c the column cross-sectional area available to the mobile phase. In a packed bed the flow of mobile phase occurs predominantly through the interparticle spaces and the mobile phase velocity at the column outlet is thus described by the equation $u_o = F_c / \pi r_c^2 \epsilon_u$ where r_c is the column radius and ϵ_u the interparticle porosity. For well-packed columns ϵ_u is about 0.4. For gas chromatography under normal operating conditions with a modest pressure drop the average and outlet velocity are simply related by $u = ju_o$, where j is the gas compressibility correction factor (see section 1.4.1). At high-pressure drops, as could exist in fast gas chromatography, the average carrier gas velocity becomes proportional to the square root of the outlet velocity [112]. For supercritical fluid chromatography the correction is more complicated, in part, because fluids behave non-ideally [113].

The mobile phase flow profile and changes in local velocity depend on the driving force used to maintain bulk flow through the separation system. These driving forces can be identified as capillary, pneumatic or electroosmotic forces. Capillary forces are responsible for the transport of mobile phase in conventional column liquid chromatography and planar chromatography. These forces are generally too weak to provide either an optimum or constant mobile phase velocity for separations using small particle sorbents. They are treated elsewhere in relation to their use in planar chromatography (section 6.3.2). For now it suffices to say that capillary-controlled flow mechanisms are unsuitable for fast and efficient chromatographic separations. Pneumatic transport of the mobile phase is commonly employed in column chromatography. The mobile phase is pressurized externally and driven through the column by the pressure gradient between the column inlet and exit. A consequence of this pressure-driven flow is a parabolic radial velocity profile, Figure 1.5, and for compressible mobile phases, a local velocity that varies with position reflecting the decreasing flow resistance with migration along the column. Electroosmosis is the source of bulk liquid flow in an electric field. At the column wall or particle surface (packed columns) an electrical double layer results from the adsorption of ions from the

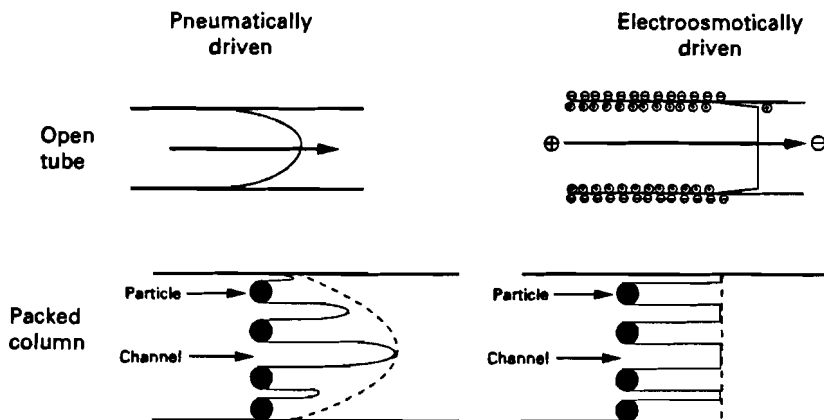


Figure 1.5. Mobile phase flow profile for an open tube and a packed column with pressure-driven and electroosmotic flow.

mobile phase or dissociation of surface functional groups. An excess of counterions is present in the double layer compared to the bulk liquid. In the presence of an electric field shearing of the solution occurs only within the microscopically thin diffuse part of the double layer transporting the mobile phase through the column with a nearly perfect plug profile, Figure 1.5. This is the general transport mechanism for neutral compounds in capillary electrophoresis, electrochromatography and micellar electrokinetic chromatography. The electrophoretic mobility of ions is also responsible for their migration in an electric field (section 8.2.1). An advantage of plug flow over parabolic flow is that it minimizes contributions from eddy diffusion to the column plate height, a major source of band broadening in pressure-driven chromatography. The electroosmotic velocity depends on a completely different set of column parameters to pressure-driven flow and for liquids provides an alternative transport mechanism to obtain efficient separation systems.

In pressure-driven flow Darcy's law provides the defining relationship between the column pressure drop and column characteristic properties. For gas chromatography, the mobile phase velocity at the column outlet is given by Eq. (1.12)

$$u_0 = KP_0(P^2 - 1) / 2\eta L \quad (1.12)$$

where K is the column permeability, P_0 the column outlet pressure, P the relative pressure (ratio of the column inlet to outlet pressure), η the mobile phase viscosity and L the column length. Eq. (1.12) is valid for open tubular columns under all normal conditions and for packed columns at low mobile phase velocities. Since liquids are largely incompressible under normal operating conditions the equivalent relationship for liquid chromatography is Eq. (1.13) [114]

$$u = \Delta PK_0 d_p^2 / \eta L \quad (1.13)$$

where ΔP is the column pressure drop, K_0 the specific permeability coefficient, and d_p the average particle diameter. Eq. (1.13) is valid for pressure drops up to about 600 atmospheres. The specific permeability coefficient has a value of about 1×10^{-3} , and can be estimated from the semi-empirical Kozeny-Carman equation [111]. The product $K_0 d_p^2$ is the column permeability. Eq. (1.12) and (1.13) are important for setting practical limits to the performance of pressure-driven chromatographic systems. Eventually inlet pressure becomes the upper bound for the achievable separation performance.

1.5.2 Rate Theories

Rather than a single defining relationship a number of similar models that encompass the same sources of band broadening but express the results in a different mathematical form are used in chromatography [12,37,103-108,115]. It was only quite recently that NMR imaging and photographic recording were used to visualize band broadening within the column packing [116-118]. Fortuitously these studies, and those performed using radial position detection, have served to demonstrate that basic theories are adequate to account for experimental observations. Interestingly, these studies have shown that dry packed and slurry packed columns exhibit different behavior with respect to radial velocity variations. Column permeability is highest in the core region for dry packed columns and at the wall for slurry packed columns. These studies confirm that velocity heterogeneity across the column radius combined with parabolic flow in pressure-driven chromatographic systems is a major cause of band broadening in packed columns.

Rate theory considers three general contributions to band broadening, which can be identified as eddy diffusion, longitudinal diffusion, and resistance to mass transfer. These contributions to the band broadening mechanism are treated as independent variables except under some circumstances when the eddy diffusion term is coupled to the mobile phase mass transfer term. The general approach can be applied to gas, liquid and supercritical fluid mobile phases, although it is necessary to make allowances for the differences in mobile phase physical properties, Table 1.5. The details of band broadening for supercritical fluid mobile phases are more complex than for gas and liquid mobile phases and to allow a simple presentation these are presented separately in section 7.4. Instrumental contributions to band broadening can be significant and are often unavoidable for miniaturized separation systems (section 1.5.4).

Eddy diffusion results from radial flow inequalities through a packed bed. The packing density for all real columns is heterogeneous in both the radial and axial direction. In addition, streamlines are not straight since molecules are forced to continually change direction by the obstacles (packing material) in their way. The local mobile phase velocity is faster through open spaces and wider interparticle spaces. The interparticle space is made up of a network of interconnected channels that experience very fast changes in cross section that depends on the particle shape and packing density. Because of parabolic flow in pressure-driven systems the mobile phase velocity is close to zero at the

Table 1.5

Characteristic values for column parameters related to band broadening

Property	Column type	Mobile phase		
		Gas	Supercritical fluid	Liquid
Diffusion coefficient (m^2/s)		10^{-1}	$10^{-4} - 10^{-3}$	10^{-5}
Density (g/cm^3)		10^{-3}	0.3 - 0.8	1
Viscosity (poise)		10^{-4}	$10^{-4} - 10^{-3}$	10^{-2}
Column length (m)	Packed	1 - 5	0.1 - 1	0.05 - 1
	Open tubular	10 - 100	5 - 25	
Column internal diameter (mm)	Packed	2 - 4	0.3 - 5	0.3 - 5
	Open tubular	0.1 - 0.7	0.02 - 0.1	< 0.01
Average particle diameter (μm)		100 - 200	3 - 20	3 - 10
Column inlet pressure (atm)		< 10	< 600	< 400
Optimum velocity (cm/s)	Packed	5 - 15	0.4 - 0.8	0.1 - 0.3
	Open tubular	10 - 100	0.1 - 0.5	
Minimum plate height (mm)	Packed	0.5 - 2	0.1 - 0.6	0.06 - 0.30
	Open tubular	0.03 - 0.8	0.01 - 0.05	> 0.02
Typical system efficiency (N)	Packed	$10^3 - 10^4$	$1 - 8 \times 10^4$	$0.5 - 5 \times 10^4$
	Open tubular	$10^4 - 10^6$	$10^4 - 10^5$	

particle surface and increases rapidly towards the center of each channel. Thus, local velocities vary strongly, depending on the size of the interstices, the proximity to a particle surface, and the continuous blocking of the flow channels by successive particles. Molecular movement through such beds is possible only when molecules avoid the obstacles by moving round them in all possible radial and axial directions. However, because of radial diffusion individual solutes are not restricted to a single streamline but sample many in their passage through the packed bed. This results in an averaging of flow inequalities that acts to minimize dispersion. This is less than perfect and the uncompensated flow inequalities result in additional band broadening that would not exist if the packing structure was ideal. In the simplest sense these variations in the flow direction and rate lead to band broadening that should depend only on the density and homogeneity of the column packing. Its contribution to the total plate height, H_E , is proportional to the average particle size, d_p , and the column packing factor, λ , expressed by $H_E = 2\lambda d_p$. The packing factor is a dimensionless constant and usually has a value between 0.5 and 1.5. Band broadening due to eddy diffusion in packed beds can be minimized by employing packings of the smallest practical particle size with a narrow particle size distribution. The column pressure drop will ultimately determine the most practical particle size and column length. For typical operating pressures this corresponds to an average particle size of about 100 μm for gas chromatography and about 3 to 5 μm for liquid chromatography. For open tubular columns the eddy diffusion term is zero because there are no particles to disrupt flow lines.

The contribution to the plate height from molecular diffusion in the mobile phase arises from the natural tendency of the solute band to diffuse away from regions of high concentration to regions of lower concentration. Its contribution to the total plate height, H_L , is proportional to the diffusion coefficient in the mobile phase, D_M , the

tortuosity (obstruction) factor of the column, γ , and the time the sample spends in the column according to $H_L = (2\gamma D_M / u)$. The obstruction factor is a dimensionless quasi-constant that is not totally independent of the mobile phase velocity. This dependence arises from the fact that the lowest flow resistance is offered by gaps or voids in the packing structure. Thus, at low velocities the value of the obstruction factor is averaged over tightly packed and loosely packed domains, while at high velocities it is weighted in favor of the loosely-packed domains where more flow occurs. Typical values for the obstruction factor are 0.6 to 0.8 in a packed bed and 1.0 for an open tubular column. Diffusion coefficients are about 10,000 times larger for gases than liquids and the contribution from longitudinal diffusion is almost always important in gas chromatography but is often negligible in liquid chromatography. Longitudinal diffusion is the principal source of band broadening in capillary electrophoresis and micellar electrokinetic chromatography (section 8.2.3).

Resistance to mass transfer is determined by the limitations of diffusion in the mobile and stationary phases as a transport mechanism to move analyte molecules to the boundary region between phases. Molecules in the vicinity of the phase boundary access the opposite phase quickly while those at a greater distance will require more time. During this time analyte molecules in the mobile phase will be transported some distance along the column. Since mass transfer is not instantaneous complete equilibrium is not established under normal separation conditions. The result is that the analyte concentration profile in the stationary phase is always displaced slightly behind the equilibrium position and the mobile phase profile is similarly slightly in advance of the equilibrium position. The combined peak observed at the column outlet is broadened about its band center, which is located where it would have been for instantaneous equilibrium, provided that the degree of non-equilibrium is small. The stationary phase contribution to resistance to mass transfer, H_S , is given by Eq. (1.14)

$$H_S = 2kd_f^2u / 3D_S(1 + k)^2 \quad (1.14)$$

where d_f is the stationary phase film thickness, and D_S the diffusion coefficient in the stationary phase. Eq. (1.14) applies exactly to thin-film open tubular columns and is a reasonable approximation for packed column gas chromatography. For liquid chromatography the agreement is poor since there is no allowance made for the contribution of slow diffusion in the stagnant mobile phase. Here, Eq (1.15) provides a more realistic model for the stationary phase mass transfer contribution to the column plate height in liquid chromatography than Eq. (1.14)

$$H_S = [\theta(k_0 + k + k_0k)^2 d_p^2 u_e] / [30D_M k_0(1 + k_0)^2(1 + k)^2] \quad (1.15)$$

where θ is the tortuosity factor for the pore structure of the particles, k_0 the ratio of the intraparticle and interparticle volumes, and u_e the interparticle mobile phase velocity. With multicomponent eluents the value of k_0 will vary with the mobile phase composition since the intraparticle space occupied by the stagnant mobile phase may

change due to solvation of the stationary phase surface. In the derivation of Eq. (1.15), the influence of diffusion through the interparticle stagnant mobile phase has been neglected as it is generally very small compared to the value for the intraparticle stagnant mobile phase contribution.

The calculation of resistance to mass transfer in the mobile phase requires an exact knowledge of the flow profile of the mobile phase. This is only known exactly for open tubular columns for which the contribution to the total plate height from resistance to mass transfer in the mobile phase, H_M , can be described by Eq. (1.16)

$$H_M = [(1 + 6k + 11k^2) / 96(1 + k)^2][d_C^2 u / D_M] \quad (1.16)$$

where d_C is the column diameter. In a packed bed the mobile phase flows through a tortuous channel system and radial mass transfer can take place by a combination of diffusion and convection. The diffusion contribution to the total plate height from resistance to mass transfer in the mobile phase, $H_{M,D}$, is given approximately by Eq. (1.17)

$$H_{M,D} = w u d_P^2 / D_M \quad (1.17)$$

where w is an empirical packing factor function used to correct for radial diffusion (ca. 0.02 to 5). To account for the influence of convection, that is, band broadening resulting from the exchange of solute between flow streams moving at different velocities, the eddy diffusion term must be coupled to the mobile phase resistance to mass transfer term, as indicated below

$$H_{MC} = 1 / (1/H_E + 1/H_{M,D}) \quad (1.18)$$

where H_{MC} is the contribution to the plate height resulting from the coupling of eddy diffusion and mobile phase mass transfer terms. In general, H_{MC} increases with increasing particle size and flow velocity and decreases with increasing solute diffusivity. The packing structure, the velocity range, and the retention factor can significantly influence the exact form of the relationship. In gas chromatography, the coupled plate height equation flattens out the ascending portion of the van Deemter curve at high mobile phase velocities in agreement with experimental observations. At flow velocities normally used the coupling concept appears to be unnecessary to account for experimental results. In liquid chromatography the existence of a coupling term and its most appropriate form is a matter that remains unsettled [37,107].

Although the above listing of contributions to the column plate height is not comprehensive, it encompasses the major band-broadening factors and the overall plate height can be expressed as their sum ($H_E + H_L + H_S + H_M$). A plot of the column plate height, H , against the mobile phase velocity is a hyperbolic function (Figure 1.6) most generally described by the van Deemter equation (1.19) [119].

$$H = A + B / u + (C_S + C_M)u \quad (1.19)$$

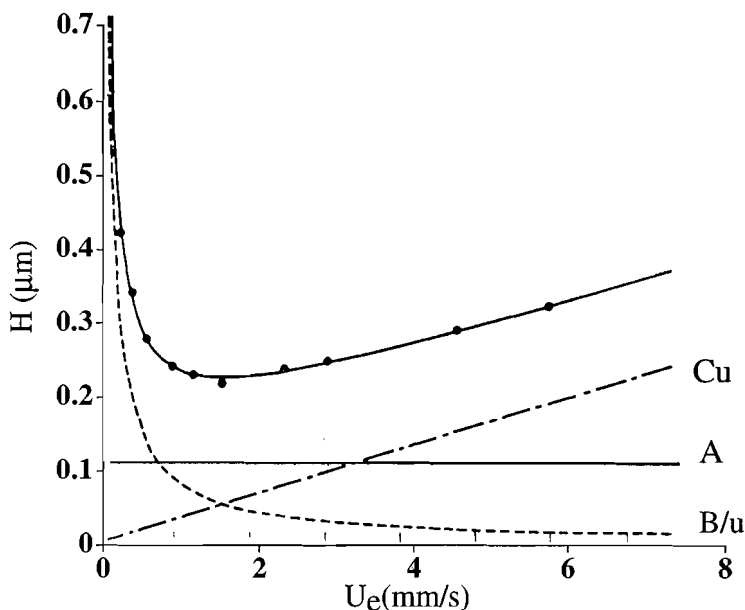


Figure 1.6. Relationship between the column plate height and mobile phase velocity for a packed column in liquid chromatography.

The *A* term represents the contribution from eddy diffusion, the *B* term the contribution from longitudinal diffusion, and the *C* terms the contributions from resistance to mass transfer in the stationary and mobile phases to the total column plate height. By differentiating equation (1.19) with respect to the mobile phase velocity and setting the result equal to zero, the optimum values of mobile phase velocity (u_{opt}) and plate height (H_{min}) can be obtained.

$$u_{opt} = [B / (C_M + C_S)]^{1/2} \quad (1.20)$$

$$H_{min} = A + 2[B(C_M + C_S)]^{1/2} \quad (1.21)$$

An important general contribution of the van Deemter equation was the illustration that an optimum mobile phase velocity existed for a column at which its highest efficiency would be realized. For less demanding separations columns may be operated at mobile phase velocities higher than u_{opt} to obtain shorter separation times. Provided that the ascending portion of the van Deemter curve is fairly flat at higher velocities than u_{opt} , then the saving of time for a small loss in efficiency is justified. From Eq. (1.21) we see that the highest efficiency of a packed column is never less than the contribution from eddy diffusion so there is no redeeming value for columns with anything but a homogenous packing structure.

Optimization of column properties with respect to band broadening comes from an understanding and interpretation of the coefficients of the van Deemter equation [105,120-123]. For gas chromatography the compressibility of the mobile phase and its influence on the local velocity and diffusion coefficients along the column must be accounted for. Rearrangement in terms of the outlet pressure and velocity aids a general interpretation that is simple to do for the eddy diffusion term, longitudinal diffusion, and the mobile phase mass transfer term. The stationary phase mass transfer term, however, cannot be expressed in an explicit form independent of the column pressure drop. At high column pressure drops, such as those that might be encountered in fast gas chromatography with narrow bore open tubular columns, modification of Eq. (1.19) was recommended to include a change in the general dependence on the mobile phase gas velocity ($H = B / u^2 + C_M u^2 + C_S u$) [124].

Beginning with the most favorable case, band broadening in open tubular columns is satisfactorily described by the Golay equation, extended to situations of appreciable pressure drop by Giddings, Eq. (1.22)

$$H = f_1[(2D_{M,o} / u_o) + (f_g(k))(d_C^2 u_o / D_{M,o})] + f_2[(f_S(k))d_f^2 u_o / D_S] \quad (1.22)$$

$$f_1 = 9/8 (P^4 - 1)(P^2 - 1) / (P^3 - 1)^2$$

$$f_2 = 3/2 (P^2 - 1) / (P^3 - 1)$$

$$f_g(k) = (1 + 6k + 11k^2) / 96 (1 + k)^2$$

$$f_S(k) = 2k / 3(1 + k)^2$$

where $D_{M,o}$ is the mobile phase diffusion coefficient at the column outlet pressure, u_o the mobile phase velocity at the column outlet, d_f the stationary phase film thickness, and P the ratio of column inlet to outlet pressure [122-126].

Open tubular columns in current use have internal diameters within the range 0.1 to 0.6 mm and a stationary phase film thickness from about 0.05 to 8.0 μm . Gases of high diffusivity, hydrogen or helium, are used to minimize mass transfer resistance in the mobile phase and, at the same time, minimize separation time (H_{\min} occurs at higher values of u_{opt} for gases of high diffusivity). Narrow bore columns are capable of higher intrinsic efficiency since they minimize the contribution from resistance to mass transfer in the mobile phase to the column plate height, Figure 1.7. The C_M term increases successively with increasing column diameter and is also influenced by the retention factor, particularly at low values of the retention factor. When combined with the term describing the plate height contribution due to longitudinal diffusion, C_M is the dominant cause of band broadening for wide bore, thin-film columns. The stationary phase mass transfer term becomes increasingly important as film thickness increases, Table 1.6 [127]. For thin-film columns ($d_f < 0.25 \mu\text{m}$) the stationary phase resistance to mass transfer term is generally only a few percent of the mobile phase

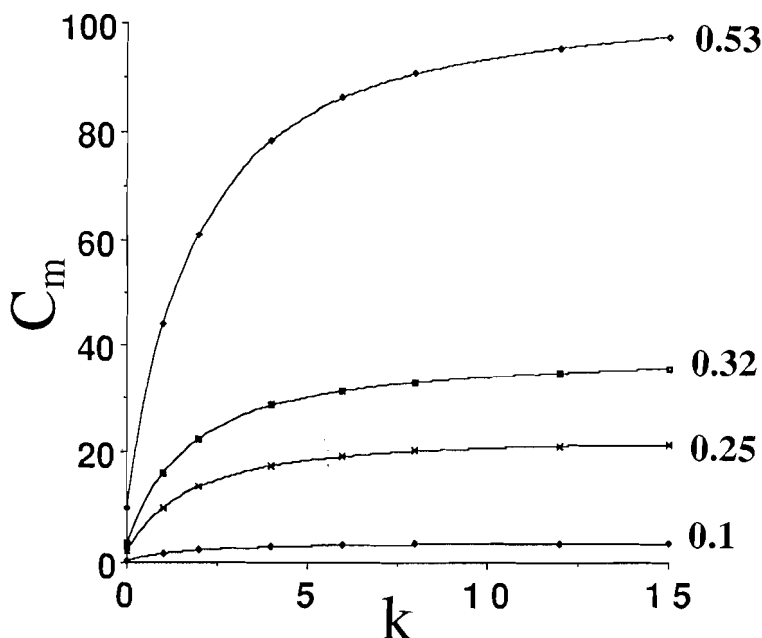


Figure 1.7. Variation of the resistance to mass transfer in the mobile phase, C_M , as a function of the retention factor for open tubular columns of different internal diameters (mm).

Table 1.6

Relative contribution (%) of resistance to mass transfer in the mobile and stationary phases to the column plate height for undecane at 130°C for a 0.32 mm internal diameter open tubular columns in gas chromatography

Film thickness (μm)	Retention factor	Phase ratio	Mass transfer term (%)	
			C_M	C_S
0.25	0.56	320	95.2	4.8
0.5	1.12	160	87.2	12.8
1.00	2.24	80	73.4	26.6
5.00	11.2	16	31.5	68.5

term and, to a first approximation, can be neglected. In estimating the contribution of stationary phase mass transfer resistance to the plate height there is a strong dependence on the retention factor and the diffusion coefficient of the solute in the stationary phase, Figure 1.8. Diffusion coefficients in polar, gum and immobilized phases tend to be smaller than those observed for other phases. Thick-film open tubular columns prepared from polar immobilized stationary phases tend to be significantly less efficient than similar columns prepared from low polarity stationary phases; the efficiency of both column types decreases with increasing film thickness.

The Golay equation can also be used to predict optimum separation conditions in open tubular column liquid chromatography [128,129]. The main difference between

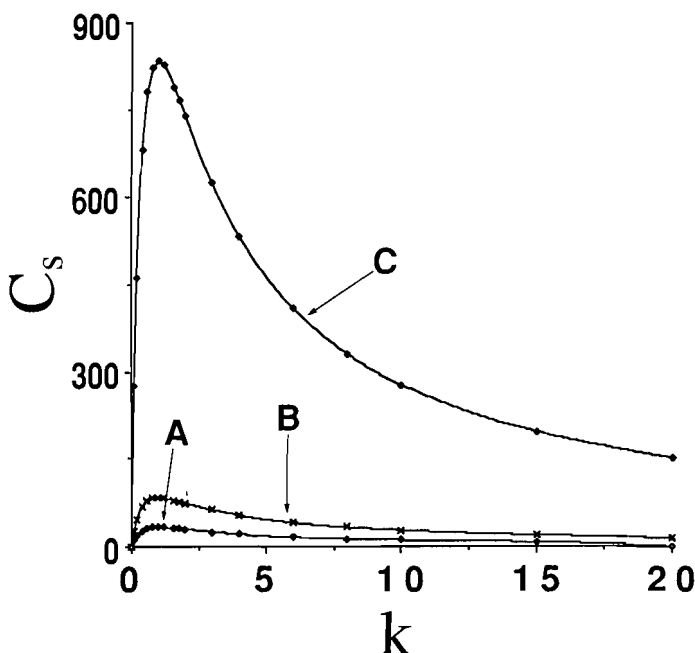


Figure 1.8. Variation of the resistance to mass transfer in the stationary phase, C_s , as a function of the retention factor for different film thicknesses. A, $d_f = 1 \mu\text{m}$ and $D_S = 5 \times 10^{-7} \text{ cm}^2/\text{s}$; B, $d_f = 5 \mu\text{m}$ and $D_S = 5 \times 10^{-6} \text{ cm}^2/\text{s}$; and C, $d_f = 5 \mu\text{m}$ and $D_S = 5 \times 10^{-7} \text{ cm}^2/\text{s}$.

gas and liquid chromatography in open tubular column is that the diffusion coefficients in liquids are roughly 10,000 times smaller than in gases and therefore the last term in Eq. (1.22) can be neglected. For high efficiency the column internal diameter must be reduced to a very small size (1-10 μm) to overcome the slow mass transfer in the mobile phase. This creates considerable instrument and column technology constraints that limit the practical utility of open tubular column liquid chromatography at present.

Since the exact flow profile of the mobile phase through a packed bed is unknown, only an approximate description of the band broadening process can be attained. For packed column gas-liquid chromatography at low mobile phase velocities, Eq. (1.23) provides a reasonable description of the band broadening process [103].

$$H = 2\lambda dp + 2\gamma D_{M,o} / u_o + [f_g(k)](dp^2 / D_{M,o})u_o + [f_s(k)](d_f^2 / D_S)u \quad (1.23)$$

According to Scott the average linear velocity can be replaced by $(4u_o / [P + 1])$ in Eq. (1.23) to permit evaluation entirely in terms of the outlet velocity [130]. If $\lambda = 0$, $\gamma = 1$, and $dp = r_c$ is substituted into Eq. (1.23) then this equation can be used as an alternative to Eq. (1.22) to account for band broadening in evaluating open tubular columns [121,130]. For gas-solid chromatography the stationary phase mass transfer

term (C_S) is replaced by C_k describing the kinetics of adsorption and desorption from a solid surface, which is often the dominant term in the plate height equation for inorganic oxide and chemically bonded adsorbents [131,132].

For packed column gas chromatography small particles with a narrow size distribution and coated with a thin, homogeneous film of liquid phase are required for high efficiency. The typical range of inlet pressures controls the absolute particle size. Column packings with particle diameters of 120-180 μm in columns less than 5 meters long are generally used with inlet pressures less than 10 atmospheres. For a liquid phase loading of 25-35% w/w, slow diffusion in the stationary phase film is the principal cause of band broadening. With lightly loaded columns (< 5% w/w), resistance to mass transfer in the mobile phase is no longer negligible. At high mobile phase velocities the coupled form of the plate height equation is used to describe band broadening.

Several equations in addition to the van Deemter equation, have been used to describe band broadening in liquid chromatography, Eqs. (1.23) to (1.26) [104,106,107,132-134].

$$H = A / [1 + (E / u)] + B / u + C u \quad (1.23)$$

$$H = A / [1 + (E / u^{1/2})] + B / u + C u + D u^{1/2} \quad (1.24)$$

$$H = A u^{1/3} + B / u + C u \quad (1.25)$$

$$H = A / [(1 + E / u^{1/3})] + B / u + C u + D u^{2/3} \quad (1.26)$$

A, B, C, D, and E are appropriate constants for a given solute in a given chromatographic system. A comparison of these equations by Katz et al. [135] indicated a good fit with experimental data for all equations, but only Eqs. (1.19), (1.23), and (1.25) consistently gave physically meaningful values for the coefficients.

The highest efficiency in liquid chromatography is obtained using columns packed with particles of small diameter, operated at high inlet pressures, with mobile phases of low viscosity. Diffusion coefficients are much smaller in liquids than in gases and, although this means that longitudinal diffusion as a source of band broadening can often be neglected, the importance of mass transfer in the mobile phase is now of greater significance. The adverse effect of slow solute diffusion in liquid chromatography can be partially overcome by operating at much lower mobile phase velocities than is common for gas chromatography. This increase in efficiency, however, is obtained at the expense of longer separation times. Slow mass transfer in the stationary phase is a result of slow diffusion through the stagnant mobile phase trapped in the pore structure and surface diffusion along the particle surface of solvated chemically bonded phases [136]. Unfavorable intraparticle mass transfer in porous particles is minimized by using particles of smaller diameter, Figure 1.9, since this restricts the average path length over which the solute must be transported by diffusion. For particles less than 5 μm in diameter the plate height curves are essentially flat in the region of the minimum value indicating

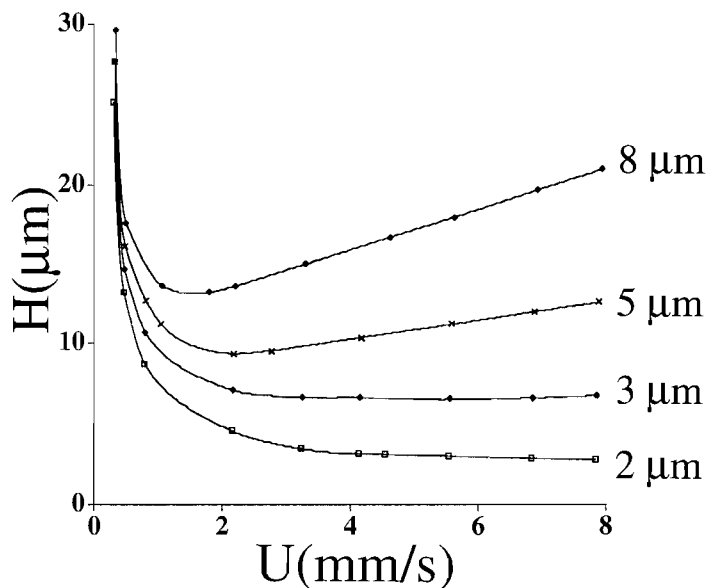


Figure 1.9. Plot of the plate height H (μm) against the mobile phase velocity u (mm/s) for columns of different particle diameters (and different column lengths) in liquid chromatography.

that small-particle diameter columns can be operated at higher linear velocities without appreciable loss in efficiency. The high flow resistance of these columns, however, prevents long columns from being used with typical inlet pressures. Nonporous particles, of course, provide an optimum means of minimizing the contribution of slow intraparticle mass transfer to column efficiency in liquid chromatography [137]. The low surface areas of these particles, however, are a disadvantage for optimizing the separation of small molecules owing to their low retention. For macromolecules this is less of a problem. The diffusion coefficients of macromolecules are one to two orders of magnitude smaller than for low molecular weight compounds. For macromolecules small diameter porous and nonporous particles yield enhanced column efficiency by virtue of the relatively small intraparticle mass transfer term (short diffusion distances) and to a lesser extent due to the small contribution of eddy diffusion to the plate height [138]. High temperatures [139] and enhanced fluidity mobile phases (prepared by adding low viscosity liquids, usually carbon dioxide, to typical mobile phases) [140,141] improve solute diffusion properties and decrease mobile phase viscosity. This results in increased efficiency and shorter separation times.

1.5.3 Reduced Parameters

The major advantage of using reduced parameters (h , v , ϕ) in place of the absolute parameters (H , u , K_0) is that results obtained from columns containing packing

materials of different sizes, operated with mobile phases of different viscosity, and evaluated with solutes of different diffusion coefficients, can be compared directly. This approach also leads to a simple index of performance, the separation impedance, which can be used to judge the relative performance of columns of a similar kind as well as the limit to performance of different column types. Reduced parameters are widely used in all types of chromatography but it was in the early development of liquid chromatography that they had their main impact on column design and testing [107,108,142-146]. Since liquids are largely incompressible the use of reduced parameters in liquid chromatography is straightforward. For compressible mobile phases it is necessary to account for the influence of the operating conditions on mobile phase viscosity and diffusion coefficients. For simplicity the examples employed in this section will be for liquid chromatography but the general approach is sound for any chromatographic technique except for those based on electrophoretic migration.

The reduced plate height, h , is defined as the number of particles to a theoretical plate and is given by

$$h = H / d_p = (1 / 5.54)(L / d_p)(w_h / t_R)^2 \quad (1.27)$$

where w_h is the peak width at half height and t_R the solute retention time. The reduced velocity, u , is the rate of flow of the mobile phase relative to the rate of diffusion of the solute over one particle diameter and is given by

$$u = u_d p / D_M = L d_p / t_M D_M \quad (1.28)$$

where D_M is the solute diffusion coefficient in the mobile phase and t_M the column holdup time. When the diffusion coefficient is unknown an approximate value can be found from the Wilke-Chang equation

$$D_M = A(\psi M_2)^{1/2} T / \eta V^{0.6} \quad (1.29)$$

where A is a constant depending on the units used (equal to 7.4×10^{-12} when D_M is in m^2/s), ψ a solvent dependent constant (1.0 for unassociated solvents, 1.5 for ethanol, 1.9 for methanol, and 2.6 for water), T the temperature in K, η the mobile phase viscosity, and V the solute molar volume [147]. For mixed solvents the volume average value for the product (ψM_2) is used [145]. Typical values for low molecular weight solutes fall into the range $0.5\text{--}3.5 \times 10^{-9} \text{ m}^2/\text{s}$. The higher value is typical of organic solvents of low viscosity such as hexane, and the lower value for polar aqueous solvents. The column flow resistance parameter, ϕ , is a measure of the resistance to flow of the mobile phase and takes into account the influence of the column length, particle diameter, and mobile phase viscosity. It is given by

$$\phi = \Delta P d_p^2 t_M / \eta L^2 \quad (1.30)$$

Table 1.7

Typical values of the reduced parameters for liquid chromatography columns

Column type	Minimum reduced plate height	Minimum reduced velocity	Flow resistance parameter	Separation impedance
Conventional packed	1.5-3.0	3-5	500-1000	2000-9000
Small-bore packed	1.5-3.0	3-5	500-1000	2000-9000
Packed capillary	2.0-3.5	1-5	350-1000	3000-7000
Open Tubular	0.5-30	4-30	32	8-80

where ΔP is the column pressure drop. Finally, the separation impedance, E , which represents the elution time per plate for an unretained solute times the pressure drop per plate, the whole corrected for viscosity is given by

$$E = (t_M / N)(\Delta P / N)(1 / \eta) = h^2 \phi \quad (1.31)$$

The separation impedance represents the difficulty of achieving a certain performance and should be minimized for optimum performance. The highest performance is achieved by a column, which combines low flow resistance and produces minimal band broadening. For open tubular columns the column internal diameter, d_c , replaces d_p in Eqs. (1.27), (1.28) and (1.30).

Some typical reduced parameter values for different liquid chromatography columns are summarized in Table 1.7. A reduced plate height of 2 is considered excellent for a packed column with more typical values for good columns lying between 2 and 3. The flow resistance parameter for packed columns will normally lie within the range 500 to 1000. It provides information on how the chromatographic system as a whole is performing. Unusual values of the flow resistance parameter are often associated with blockages in the system (frits, connecting tubing, etc.), packings containing fine particles, or columns containing excessive voids. The separation impedance then has values between 2000 and 9000 for good columns. The performance of packed columns should be independent of the column diameter and this is reflected in the similar values for the separation impedance for conventional, small bore, and packed capillary columns. Slightly lower plate heights and reduced flow resistance have been observed for packed capillary columns with low aspect ratios (ratio of column diameter / particle diameter) [148-150]. This is most likely due to the influence of the column wall on the packing density resulting in decreased flow dispersion and a reduction in the mobile phase mass transfer contribution to the plate height.

According to chromatographic theory, the reduced plate height is related to the reduced velocity by Eq. (1.32)

$$h = A v^{1/3} + B / v + C v \quad (1.32)$$

The constant B reflects the geometry of the mobile phase in the column and the extent to which diffusion of the solute is hindered by the presence of the packing. For columns

yielding acceptable fits to Eq. (1.32), B is expected to lie between 1 and 4, and for small molecules is typically around 2. It is responsible for the decrease in efficiency at very low flow rates. The constant A is a measure of the uniformity and density of the column packing. A well-packed column will have a value of A between 0.5 and 1.0 while a poorly packed column will have a higher value, say between 2 and 5. The constant C reflects the efficiency of mass transfer between the stationary phase and the mobile phase. At high reduced mobile phase velocities the C term dominates the reduced plate height value, and therefore column efficiency. The value for C is close to zero for a nonporous sorbent, a reasonable value is 0.003, but has a greater value for silica-based porous sorbents with a value of 0.05 being reasonable for the latter. Porous polymers may have significantly higher values approaching unity.

The constant A , B , and C can be determined by curve fitting from a plot of the reduced plate height against the reduced velocity [145,151-153]. Accurate values for the constants are only obtained if a wide range of reduced velocity values is covered and the data are of high quality. From the shape of a log-log plot of reduced plate height against reduced velocity, Figure 1.10, the important features of the column are easily deduced. If the minimum is below 3 and occurs in the range $3 < u < 10$ then the column is well packed (low A). If the reduced plate height is below 10 at a reduced velocity of 100 then the material has good mass transfer characteristics (low C) and is probably well packed as well (low A). If the curve has a high and flat minimum the column is poorly packed (high A).

Because of the time required to develop sufficient data points to make a plot similar to that shown in Figure 1.10 it is useful to have a shorter method for assessing potential problems. For a good column the value of the reduced plate height should not exceed 3 or 4 at a reduced velocity of about 5 and 10 to 20 at a reduced velocity of about 100.

The possibility of obtaining significant improvements in performance by using open tubular columns in liquid chromatography is clearly illustrated by the values for the separation impedance in Table 1.7. The dependence of the reduced plate height of an open tubular column on the reduced velocity is expressed by Eq. (1.33), assuming that the resistance to mass transfer in the stationary phase can be neglected

$$h = H / d_c = (2 / u) + [(1 + 6k + 11k^2) / 96(1 + k)^2]u \quad (1.33)$$

The reduced plate height is reasonably constant, independent of the retention factor for a packed column, while Eq. (1.33) indicates that the reduced plate height, at least for small values of the retention factor, will increase with the retention factor for open tubular columns. Actual values of the minimum reduced plate height will vary from about 0.3 (corresponding to $k = 0$) to 1.0 (corresponding to $k = \infty$) for values of the reduced velocity in the range 4-14 [142]. The exceptional potential performance of open tubular columns cannot be explained entirely by the smaller values of the reduced plate height. The most significant difference compared to a packed column is their greater permeability. The column flow resistance parameter can be calculated directly from the Poiseuille equation and is exactly 32 for open tubular columns [143]. The separation

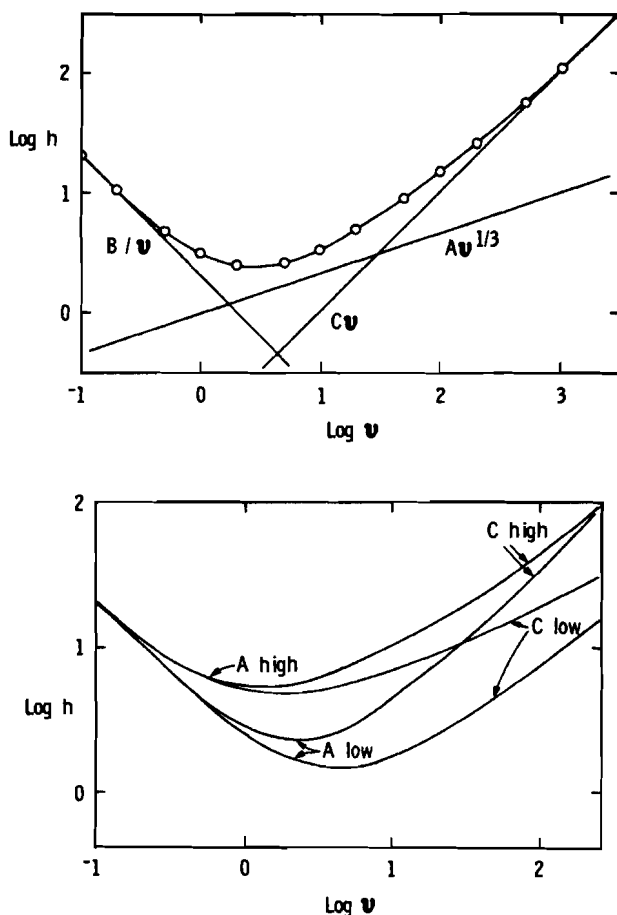


Figure 1.10. Plot of the reduced plate height against the reduced mobile phase velocity indicating the velocity region in which the three terms in Eq. (1.31) contribute to the reduced plate height. The lower figure shows some typical results from column testing. The lowest curve (A and C low) illustrates ideal behavior. The plots for other columns can be considered to deviate from this curve due to high A (poorly packed column), high C (poor quality column packing), or from having both a high A and C value.

impedance is about two orders of magnitude lower for an open tubular column compared to a packed column. If operation at the minimum separation impedance was possible then for a separation requiring a fixed number of plates the open tubular column would be about 100 times faster at a constant inlet pressure

The optimum operating conditions for the various column types are summarized in Table 1.8 [142]. There are no serious problems when operating conventional packed columns under optimum conditions, at least for particles $> 2 \mu\text{m}$. Small bore packed columns place greater demands on instrumentation but again it should be feasible to

Table 1.8

Optimum operating conditions for different columns in liquid chromatography

 $\Delta P = 200$ bar, $\eta = 1 \times 10^{-3}$ N.s/m² and $D_M = 1.0 \times 10^{-9}$ m²/s

Column plate count	Hold-up time (s)	Particle or column diameter (μ m)	Length (m)	Flow rate (μ L/s)	Peak standard deviation k = 0 (μ L)
Conventional Packed Columns: $d_C = 5$ mm, $\phi = 500$; $h_{min} = 2$; $v_{opt} = 5$; $E_{min} = 2000$; $\epsilon_T = 0.75$					
10,000	10	1.6	0.03	50	5
30,000	90	2.7	0.17	30	15
100,000	1,000	5	1	15	50
300,000	9,000	9	5	9	150
1,000,000	100,00	16	3	5	500
Small Bore Packed Columns: $d_C = 1$ mm, $\phi = 500$; $h_{min} = 2$; $v_{opt} = 5$; $E_{min} = 2000$; $\epsilon_T = 0.75$					
10,000	10	1.6	0.03	1.9	0.2
30,000	90	2.7	0.17	1.1	0.6
100,000	1,000	5	1	0.6	2
300,000	9,000	9	5	0.35	6
1,000,000	100,000	16	30	0.2	20
Open Tubular Columns: $\phi = 32$; $h_{min} = 0.8$; $v_{opt} = 5$; $E_{min} = 20$					
10,000	0.1	0.25	0.002	1.0×10^{-6}	1.0×10^{-9}
30,000	0.9	0.43	0.01	1.6×10^{-6}	0.8×10^{-8}
100,000	10	0.8	0.065	3.0×10^{-6}	1.0×10^{-7}
300,000	90	1.4	0.33	5.0×10^{-6}	0.8×10^{-6}
1,000,000	1,000	2.5	2	1.0×10^{-5}	1.0×10^{-5}
3,000,000	9,000	4.3	10	1.6×10^{-5}	8.0×10^{-5}
10,000,000	100,000	8	63	3.0×10^{-5}	1.0×10^{-3}

meet the requirements for operation under optimum conditions for plate counts in excess of 10,000. There is virtually no possibility of operating open tubular columns under optimum conditions since the eluted peak volumes for the unretained solute are so extremely small, even for high plate counts. Extracolumn contributions to dispersion should be about one-third of the volume standard deviation of an unretained solute to avoid a significant loss in performance for early eluting peaks. For an open tubular column exhibiting more than 1 million plates this corresponds to an extracolumn volume of less than 10 picoliters. Also, in practice, it may be difficult to achieve the optimum column internal diameter which for a column exhibiting 1 million plates is about 2.5 μ m. The data in Table 1.8 were compiled at a fixed pressure of 200 bar. Increasing the inlet pressure reduces the separation time but leads to smaller optimum dimensions for the column diameter and length. This in turn leads to a smaller value for the peak standard deviation and a more hopeless case in terms of the practicality of operating open tubular columns in liquid chromatography at the minimum separation impedance.

Since packed columns can be operated at $E_{min} = 2000$ with plate numbers from 10,000 to 1 million, alternative column types will compete effectively only if they can be operated at separation impedance values below 2000. Enlarging the column

Table 1.9

Detector limited operation of open tubular columns in liquid chromatography

 $\eta = 10^{-3} \text{ N.s/m}^2$; $D_M = 10^{-9} \text{ m}^2/\text{s}$; $\Delta P = 200 \text{ bar}$; $\phi = 32$; mass transfer coefficient = 0.08

Column plate count	Column hold-up time (s)	Column length (m)	Reduced plate height	Reduced velocity	Separation impedance
$d_C = 27 \text{ }\mu\text{m}$ and peak standard deviation ($k = 0$) = 0.1 μl					
10,000	600	17	64	797	130,000
30,000	1,800	30	37	464	44,000
100,000	6,000	55	20	252	13,000
300,000	18,000	90	11	140	4,000
1,000,000	60,000	170	6.4	80	1,300
$d_C = 9 \text{ }\mu\text{m}$ and peak standard deviation ($k = 0$) = 1 nl					
10,000	60	1.7	18.5	232	11,000
30,000	180	3	11.2	140	4,000
100,000	600	5.5	6.1	77	1,200
300,000	1,800	9	3.4	42	360
1,000,000	6,000	17			120

diameter and using higher reduced velocities will increase the separation impedance for the column, but still below 2000, while simultaneously increasing peak volumes to relax the instrumental constraints for column operation. Optimization under conditions where extracolumn volumes are limiting can be handled by setting a minimum value for the peak standard deviation of an unretained solute. A total instrument dispersion due to extracolumn volumes of about 0.1 μl could probably be achieved whereas 1 nl or smaller would certainly be more desirable. Considering the above considerations the separation impedance can be calculated for various sets of practical operating conditions as shown in Table 1.9. For extracolumn volume peak standard deviations of 0.1 μl an open tubular column will only show superiority over a packed column operated under optimum conditions when the plate count is in excess of about 500,000. With a further reduction to 1 nl the open tubular column is superior to the packed column for a plate count in excess of about 70,000. Consequently, the future of open tubular column liquid chromatography will depend primarily on the development of new instrument concepts with injector and detector volumes reduced to the nl level or less. Also, new column technology that permits the fabrication of narrow bore columns with sufficient stationary phase to provide reasonable partition ratios will be required. This is not a trivial accomplishment and it would seem unlikely that open tubular columns will be widely used in analytical laboratories at any time in the near future [154,155].

1.5.4 Extracolumn Sources

Under ideal conditions, the peak profile recorded during a separation should depend only on the operating characteristics of the column and should be independent of

the instrument in which the column resides. Under less than ideal conditions, the peak profile will be broader than the column profile by an amount equivalent to the extracolumn band broadening. This broadening results from the volumetric dispersion originating from the injector, column connecting tubing and detector together with the temporal dispersion resulting from the slow response of the electronic circuitry of the detector and data recording device. The various contributions to extracolumn band broadening can be treated as independent factors, additive in their variances, according to Eq. (1.34) [156-161]

$$\sigma^2_T = \sigma^2_{col} + \sigma^2_{inj} + \sigma^2_{con} + \sigma^2_{det} + \sigma^2_{tc} \quad (1.34)$$

where σ^2_T is the total peak variance observed in the chromatogram, σ^2_{col} the peak variance due to the column, σ^2_{inj} the variance due to the volume and geometry of the injector, σ^2_{con} the variance due to connecting tubes, unions, etc., σ^2_{det} the variance due to the volume and geometry of the detector, and σ^2_{tc} the variance due to the finite response time of the electronic circuits of the detector and data system. For experimental evaluation Eq. (1.34) can be simplified to $\sigma^2_T = \sigma^2_{col} + \sigma^2_{ext}$ where σ^2_{ext} is the sum of all extracolumn contributions to the peak variance and represents the instrumental contribution to band broadening. The column contribution to the peak variance can be written in time or volume units, $\sigma^2_{col} = (t_R^2 / N) = (V_R^2 / N)$ where t_R and V_R are the retention time and volume, respectively, for a peak, and N the true column efficiency in the absence of extracolumn band broadening. A commonly accepted criterion for the instrumental contribution to band broadening is that this should not exceed 10% of the column variance. This corresponds to a loss of 10% of the column efficiency and about 5% of the column resolution. A peak eluting from a column will occupy a volume equivalent to 4σ units. The above criterion can then be used to establish working limits for the acceptable extracolumn variance and volume for typical separation conditions using the properties of an unretained peak as the worst case or limiting condition.

Band broadening due to injection arises because the sample is introduced into the column as a finite volume over a finite time. A solute zone is formed at the column head, which reflects the degree of sample axial displacement during the injection time. This solute zone is generally less than the injection volume due to retention of the solute by the stationary phase. In liquid and supercritical fluid chromatography the elution strength of the injection solvent and the effect of solvent dilution with the mobile phase will determine the extent of the zone displacement. This situation is too complex to be described by a simple mathematical model. Two extreme views, those of plug or exponential injection, can be used to define the limiting cases. Valve injection occurs largely by displacement (plug injection) accompanied by various contributions from exponential dilution (mixing). The variance due to the injection profile is described by $\sigma^2_{inj} = V_{inj}^2 / K$ where V_{inj} is the injection volume and K is a constant with values between 1-12 depending on the characteristics of the injector [156,159,162,163]. For plug injection $K = 12$; more typical values for K under conditions of valve injection in liquid chromatography are 2-8.

Band dispersion in open tubes is due to poor radial mass transfer of the solute resulting from the parabolic velocity profile that exists in cylindrical tubes. The solute contained in the mobile phase close to the wall is moving very slowly and that at the center at the maximum velocity. This range of solvent velocity from the wall of the tube to the center causes a significant increased dispersion of any solute band passing through it. The variance due to connecting tubes is given by

$$\sigma_{\text{con}}^2 = \pi r_c^4 L / 24 D_M F \quad (1.35)$$

where r_c is the tube radius, L the tube length, D_M the solute diffusion coefficient in the mobile phase and F the column flow rate [106,157,161]. The dispersion in connecting tubes can be minimized by using short lengths of tubing with small internal diameters. Tubing with too small a diameter, however, increases the risk of plugging, and tubing of too short a length limits the flexibility for spatial arrangement of the instrument modules. Serpentine tubing, which introduces radial convection to break up the parabolic flow by reversing the direction of flow at each serpentine bend, is a practical alternative [164,165]. Dispersion in a serpentine tube is about 20% of that for a straight tube and allows longer connecting tubes to be used. Strictly speaking Eq. (1.35) requires a certain minimum efficiency to be an accurate description of the band broadening process. This minimum efficiency may not be reached in all cases, and the use of Eq. (1.35) may over estimate the connecting tube contribution to band broadening [166].

Depending on the design of the detector cell, it can have either the properties of a tube with plug flow or act as a mixing volume. In practice, most detector cells behave in a manner somewhere in between these two extreme models. When plug flow is dominant the variance can be calculated from $\sigma_{\text{det}}^2 = V_{\text{det}}^2 / 12$ and when mixing dominates from Eq. (1.35) by substituting the appropriate terms for the detector cell radius and length. Generally, if the cell volume is approximately 10% of the peak volume, then extracolumn band broadening from the detector will be insignificant. Higher flow rates through the detector cell result in a decrease in the peak residence time accompanied by a smaller contribution from extracolumn band broadening at the expense of sensitivity. Detectors and data systems can also cause band broadening due to their response time, which is primarily a function of the time constant associated with the filter network used to diminish high frequency noise. With the exception of fast chromatography the contribution of the electronic response time to band broadening should not be significant for modern instruments.

There are two general experimental methods for estimating the extracolumn band broadening of a chromatographic instrument. The linear extrapolation method is relatively straightforward to perform and interpret but rests on the validity of Eq. (1.34) and the model used to calculate the contribution for the column variance. A plot of σ_T^2 against t_R^2 , V_R^2 or $(1+k)^2$ for a series of homologous compounds will be linear. The true column efficiency can be obtained from the slope of the line and σ_{ext}^2 from the intercept on the vertical axis [162,167,168]. The assumption that the individual

contributions to the extracolumn variance are independent may not be true in practice, and it may be necessary to couple some of the individual contributions to obtain the most accurate values for the extracolumn variance [156]. The calculation of the column contribution assumes that solute diffusion coefficients in the mobile and stationary phases are identical and that any variation in the column plate height, as a function of the retention factor, can be neglected [168]. These assumptions are unlikely to be true in all cases diminishing the absolute accuracy with which extracolumn variance can be calculated using the linear extrapolation method. The zero length column method uses a short length of connecting tubing to replace the column [106,157]. It is then assumed that the observed total dispersion for an injected peak arises from system components only. This approach is experimentally demanding and ignores the contribution from column connecting fittings to the extracolumn variance.

1.5.5 Isotherm Effects

For separation conditions pertaining to analytical chromatography it is often assumed that the distribution constant is independent of the analyte concentration and a plot of the analyte concentration in the stationary phase against its concentration in the mobile phase is linear with a slope equal to the distribution constant. Such a plot is called an isotherm because it is obtained at a single temperature. The resultant peak shape is symmetrical with a width dependent on the kinetic properties of the column. This is the basis of linear chromatography on which the general theory of chromatography is constructed. Nonlinear isotherms are observed under certain circumstances and result in peak asymmetry and retention times that depend on the concentration of analyte in the mobile phase, and therefore the injected sample sizes [27,169]. Nonlinear isotherms are common in preparative-scale chromatography due to the use of large sample sizes to maximize yield and production rate [12].

Common causes of nonlinear isotherms are high sample concentrations and energetically heterogeneous adsorbents containing adsorption sites with incompatible association/dissociation rate constants [12,21,169-171]. Chemically bonded phases used in liquid chromatography, for example, contain sorption sites associated with the surface bonded ligands and accessible silanol groups of the silica matrix [171-174]. The interaction of these sites with hydrogen-bonding solutes is likely to be different. Inorganic oxide sorbents used in gas-solid chromatography are also intrinsically heterogeneous [170]. For differences that are not too extreme experimental isotherms for monolayer coverage of solutes with strong solute-stationary phase interactions and weak solute-solute interactions can be fit to a Langmuir-type model, Eq. (1.36)

$$Q = aC / (1 + bC) \quad (1.36)$$

where Q is the analyte concentration in the stationary phase, C the analyte concentration in the mobile phase and a and b are experimental constants. The a constant is related to the distribution constant for the analyte at infinite dilution and the b constant the sorbent

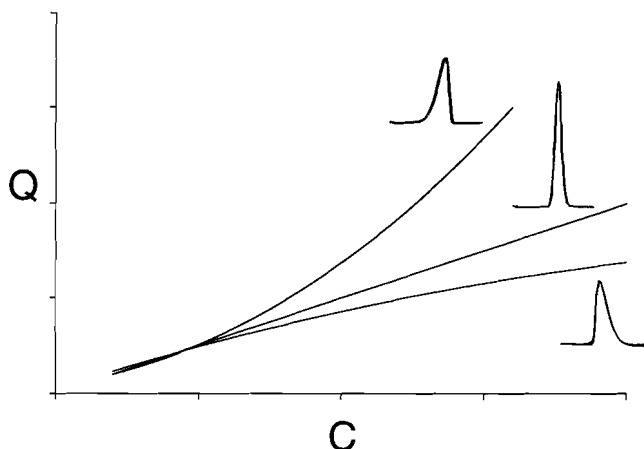


Figure 1.11. Schematic representation of different isotherms and their influence on chromatographic peak shape. Q is the analyte concentration in the stationary phase and C the analyte concentration in the mobile phase.

saturation capacity. For isotherms that do not fit Eq. (1.36) alternatives include the bi-Langmuir, Freundlich, or quadratic models [12,170,176]. In the case of a Langmuir-type isotherm, the number of unoccupied sorption sites on the stationary phase are rapidly reduced as the analyte concentration increases and the slope of the isotherm decreases, Figure 1.11. As a result, at higher analyte concentrations the band moves faster than at lower concentrations and the peak develops a tail at its rear.

Anti-Langmuir type isotherms are more common in partition systems where solute-stationary phase interactions are relatively weak compared with solute-solute interactions or where column overload occurs as a result of large sample sizes. In this case, analyte molecules already sorbed to the stationary phase facilitate sorption of additional analyte. Thus, at increasing analyte concentration the distribution constant for the sorption of the analyte by the stationary phase increases due to increased sorption of analyte molecules by those analyte molecules already sorbed by the stationary phase. The resulting peak has a diffuse front and a sharp tail, and is described as a fronting peak.

A number of experimental techniques have been described for the determination of isotherms based on frontal analysis, frontal analysis by characteristic point, elution by characteristic point, and perturbation methods [12,21,27,169,176-179]. Most authors report single-component isotherm results. Multiple-component isotherm data are more complicated because all components are simultaneously in competition for the sorption sites on the stationary phase. The retention time and peak shapes of any solute is dependent on the concentration and properties of all other solutes in the mixture [12,170,180]. For multicomponent mobile phases in liquid and supercritical fluid chromatography this includes each component of the mobile phase.

1.5.6 Peak Shape Models

Real chromatographic peaks are rarely truly Gaussian and significant errors can result from the calculation of chromatographic parameters based on this false assumption [153,164,165]. The Gaussian model is only appropriate when the degree of peak asymmetry is slight. Peak asymmetry can arise from a variety of instrumental and chromatographic sources. Those due to extracolumn band broadening (section 1.5.4) and isotherm effects (1.5.5) were discussed earlier. Other chromatographic sources include incomplete resolution of sample components, slow mass transfer processes, chemical reactions, and the formation of column voids [171, 186]. Examples of slow mass transfer processes include diffusion of the solute in microporous solids, polymers, organic gel matrices, and deep pores holding liquid droplets; interactions involving surfaces with a heterogeneous energy distribution; and, in liquid chromatography, interfacial mass transfer resistance caused by poor solvation of bonded phases. Column voids formed by bed shrinkage are usually a gradual process that occurs during the lifetime of all columns and results in progressive peak broadening and/or distortion. A void over the entire cross section of the column near the inlet produces more peak broadening than asymmetry. However, voids occupying only part of the cross section along the length of the bed can produce pronounced tailing or fronting, or even split all peaks into resolved or unresolved doublets. Partial void effects are due to channeling, that is, different residence times in the flow paths formed by the void and packed regions. Slow diffusion in liquids, fails to relax the radial concentration profile fast enough to avoid asymmetry or split peaks. In gas chromatography the phenomenon is less significant because diffusion in gases is much faster.

Meaningful chromatographic data can be extracted from asymmetric peaks by digital integration or curve fitting routines applied to the chromatographic peak profile. The statistical moments of a chromatographic peak in units of time are defined by Eq. (1.37) to (1.39) [153,184,187,188]

$$\text{Zeroth moment} \quad M_0 = \int_0^{\infty} h(t)dt \quad (1.37)$$

$$\text{First moment} \quad M_1 = (1 / M_0) \int_0^{\infty} t h(t)dt \quad (1.38)$$

$$\text{Higher moments} \quad M_n = (1 / M_0) \int_0^{\infty} (t-M_1)^n h(t)dt \quad (1.39)$$

where $h(t)$ is the peak height at time t after injection. The zeroth moment corresponds to the peak area, the first moment corresponds to the elution time of the center of gravity of the peak (retention time), and the second moment the peak variance. The column plate count is calculated from the first two moments using $N = M_1^2 / M_2$. The third and forth statistical moments measure the peak asymmetry (skew) and the extent of vertical flattening (excess), respectively. For a Gaussian distribution, statistical moments higher than the second have a value of zero. A positive value for the skew indicates a tailing peak. A positive value for the excess indicates a sharpening of the peak profile relative

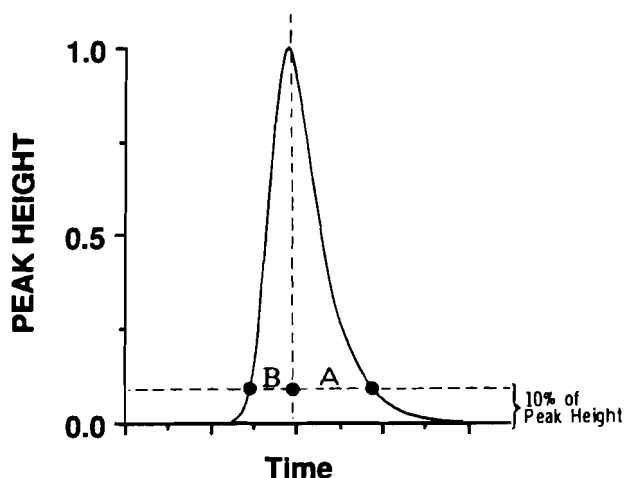


Figure 1.12. The 10% peak height definition of the asymmetry factor. The asymmetry factor is equal to the ratio A / B .

to a Gaussian peak, while a negative value indicates a relative flattening of the upper portion of the peak profile.

Direct numerical integration of the peak profile may lead to many errors and uncertainties arising from the limits used in the integration, baseline drift, noise, and extracolumn dispersion. A slight error in determining the baseline will greatly influence the selected positions for the start and end of the peak resulting in a comparatively large error, particularly for the higher moments. To eliminate these inconsistencies curve fitting of peak profiles by computer or manual methods have been explored [183,184,188-195]. This has led to the general acceptance of the exponentially modified Gaussian (EMG) and exponential Gaussian hybrid functions [194,195] as acceptable models for tailing peaks. The (EMG) is obtained by the convolution of a Gaussian function and an exponential decay function that provides for the asymmetry in the peak profile. The EMG function is defined by three parameters: the retention time and standard deviation of the parent Gaussian function and the time constant of the exponential decay function. The convolution of different functions for the peak front and tail [192,193] and method of data processing [188] increase the flexibility and improve precision, respectively. The column plate count for tailed peaks, N_{sys} , can be estimated from the chromatogram, Eq. (1.40), based on the properties of the EMG function for peaks with $1.1 < (A / B) < 2.76$.

$$N_{\text{sys}} = [41.7 (t_R / w_{0.1})^2] / [(A / B) + 1.25] \quad (1.40)$$

The width at 10% of the peak height ($w_{0.1} = A + B$) and the asymmetry function (A / B) are defined as indicated in Figure 1.12. The percent relative error between Eq. (1.40) and the EMG function was $< 2\%$. Another use of the EMG function is to indicate the

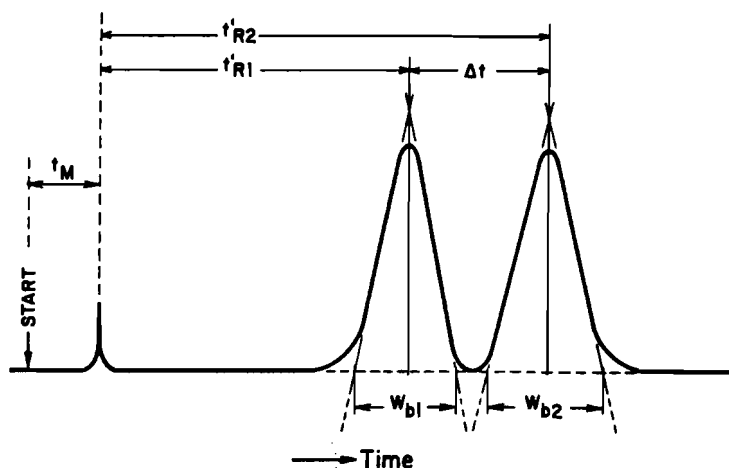


Figure 1.13. Illustration of parameters used to measure the resolution between two neighboring peaks in a chromatogram using Eq. (1.41).

magnitude of extracolumn dispersion assuming that the column behaves as a Gaussian operator [196,197].

1.6 RESOLUTION

The separation factor (α) is a useful measure of relative peak position in the chromatogram (section 1.4). This function, however, is not adequate to describe peak separations since it does not contain any information about peak widths. The separation of two peaks in a chromatogram is defined by their resolution, R_S , the ratio between the separation of the two peak maxima (Δt) and their average width at base, Figure 1.13, and Eq. (1.41)

$$R_S = 2\Delta t / (w_{b1} + w_{b2}) \quad (1.41)$$

This equation is correct for symmetrical peaks and is easily transposed to use the peak width at half height (w_h) when the peak width at base cannot be evaluated for overlapping peaks [$R_S = 1.18\Delta t / (w_{h1} + w_{h2})$]. For two peaks of similar height $R_S = 1.0$ corresponds to a valley separation of about 94% and is generally considered an adequate goal for an optimized separation. Baseline resolution requires $R_S \approx 1.5$. For symmetrical peaks of unequal height and asymmetric peaks a larger value of R_S is required for an acceptable separation as illustrated by the peak ratios in Figure 1.14 [198]. For relatively simple mixtures it is not unusual to set a higher minimum resolution requirement (e.g., $R_S > 2$) in method development to ensure ruggedness as column properties deteriorate.

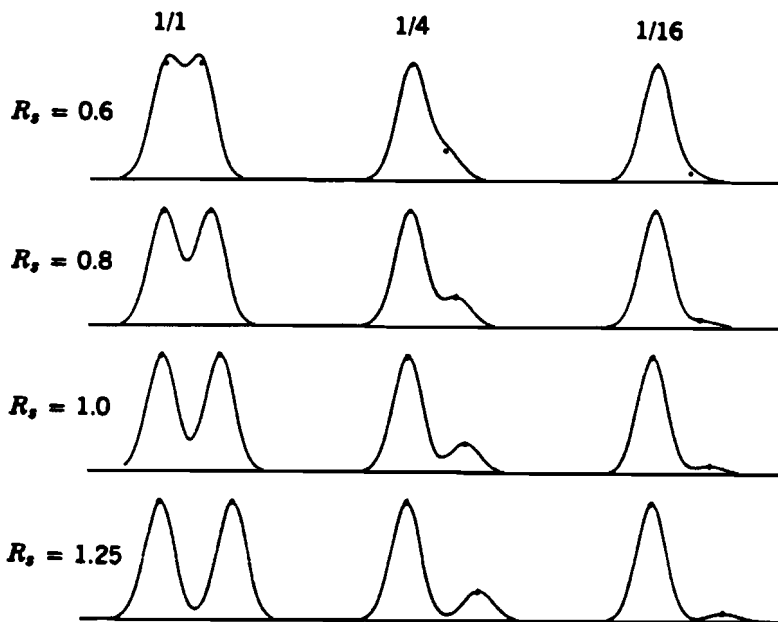


Figure 1.14. Standard resolution curves for the separation of two neighboring peaks as a function of resolution and relative peak area. (From ref [198]; ©John Wiley & Sons)

1.6.1 Relationship to Column Properties

A number of equations have been evoked to relate the resolution of two neighboring symmetrical peaks to the adjustable chromatographic variables of selectivity, efficiency, and time [198-202]. The most accurate of these assuming a similar value of N for the two peaks is

$$R_S = [\sqrt{N} / 2][(\alpha - 1) / (\alpha + 1)][k_{AV} / (1 + k_{AV})] \quad (1.42)$$

where α is the separation factor and $k_{AV} = (k_1 + k_2) / 2$ where k_1 and k_2 are the retention factors of the earlier and later eluting peaks, respectively. For computer-aided method development Eq. (1.42) can be rewritten as Eq. (1.43) by replacing α and k_{AV} with the appropriate individual retention factors

$$R_S = [\sqrt{N} / 2][(k_2 - k_1) / (2 + k_1 + k_2)] \quad (1.43)$$

Probably the most widely quoted of the resolution equations is Eq. (1.44) derived with the assumption that the average of the two peak widths is identical to the peak width of the second peak

$$R_S = [\sqrt{N} / 4][(\alpha - 1) / \alpha][k_2 / (1 + k_2)] \quad (1.44)$$

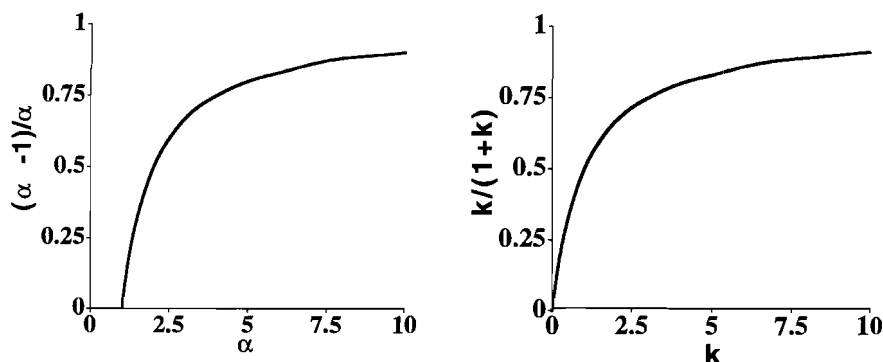


Figure 1.15. Influence of the separation factor and retention factor on the observed resolution for two closely spaced peaks.

Eq. (1.44) should be viewed as a special case of Eq. (1.42) and will tend to underestimate R_S when N is small ($< 20,000$) and will be increasingly inaccurate for large values of Δt .

To a first approximation the three terms in Eqs. (1.42) and (1.44) can be treated as independent variables. Eq. (1.44) is sufficiently sound to demonstrate the influence of the column properties on resolution and their optimization. The influence of the separation factor and retention factor on the observed resolution is illustrated in Figure 1.15 for a fixed value of N . For a separation factor of 1.0 there is no possibility of a separation. The separation factor is determined by the distribution constants for the solutes, and in the absence of some difference in distribution constants, and therefore α , there will be no separation. Increasing the value of α initially causes a large change in resolution that levels off for large values of α . For $\alpha > 2$ separations are easy. Since the separation factor has a large effect on the ease of achieving a certain resolution it is important to choose a chromatographic system that maximizes the separation factor so that the separation can be achieved with the minimum value of N and/or the shortest separation time.

Also from Figure 1.15 it can be seen that resolution is impossible without retention. Initially resolution increases rapidly with retention for $k > 0$. By the time the retention factor reaches a value of around 5, further increases in retention result in only small changes in resolution. The optimum resolution range for most separations occurs for k between 2 and 10. Higher values of k result in excessive separation time with little concomitant improvement in resolution. On the other hand, large values of k do not result in diminished resolution and may be required by necessity for the separation of multicomponent mixtures to accommodate all of the sample components in the separation.

The kinetic properties of columns are the most predictable but the least powerful for optimizing resolution. Resolution only increases with the \sqrt{N} . Increasing the column length four-fold will only double resolution at the expense of a four-fold increase in

Table 1.10

Plate count required for $R_S = 1$ for different separation conditions based on Eq. (1.44)

Retention factor	Separation factor	N_{req}	Retention factor	Separation factor	N_{req}
3	1.005	1,150,000	0.1	1.05	853,780
3	1.01	290,000	0.2	1.05	254,020
3	1.02	74,000	0.5	1.05	63,500
3	1.05	12,500	1.0	1.05	28,200
3	1.10	3,400	2.0	1.05	15,800
3	1.20	1,020	5.0	1.05	10,160
3	1.50	260	10	1.05	8,540
3	2.00	110	20	1.05	7,780

separation time and an increase in the column pressure drop. In liquid and supercritical fluid chromatography even a four-fold increase in N may be difficult to achieve if standard column configurations are used. Reducing the column diameter in open tubular column gas and supercritical fluid chromatography and the particle size in packed column liquid and supercritical fluid chromatography at a constant column length is a more effective strategy for increasing resolution than increasing the column length. This is somewhat restricted by the increased column pressure drop for liquid and supercritical fluid chromatography while there is greater flexibility in gas chromatography, where this is a more practical strategy.

Equation (1.44) is simply rearranged to predict the plate number required, N_{req} , to give a certain separation, Eq. (1.45).

$$N_{\text{req}} = 16 R_S^2 [\alpha / (\alpha - 1)]^2 [k_2 / (1 + k_2)]^2 \quad (1.45)$$

The plate count required for a resolution of 1.0 using different separation conditions is summarized in Table 1.10 [146]. Practically all chromatographic separations have to be made in the efficiency range of 10^3 - 10^6 theoretical plates. The importance of optimizing the separation factor and retention factor to obtain an easy separation is obvious from the data in Table 1.10. Easy separations require chromatographic systems that maximize the separation factor and provide at least a minimum value for the retention factor. A common optimization strategy for difficult separations with a limited number of components is to fix the value of the retention factor between 1 and 3 for the two components most difficult to separate in the mixture.

1.6.2 Objective Functions

Objective functions are used primarily for computer ranking of separation quality in approaches for automated method development. For this purpose the separation quality throughout the chromatogram must be expressed by a single-valued and easily calculated mathematical function [202-209]. This has proven to be a difficult problem and no universal solution has emerged. There is no straightforward manner to uniquely define the resolution of all peaks simultaneously by a single number. More likely, for

any given value of the objective function there will be a large number of separations that could produce the same numerical value, not all of which will agree with the stated goals of the separation. In addition, the objective function may have to consider the number of peaks identified in the chromatogram and the separation time in expressing the goals of the separation. A few representative objective functions and their relative merits are discussed below.

A simple objective function would consider only the separation between the worst separated peak pair, ignoring all others. If a set of chromatograms is to be compared, then this is a reasonable approach, but it does not provide a suitable criterion for locating a single global optimum, since different peaks may show the lowest separation in adjacent chromatograms and many optima will be indicated. The sum of all the resolutions will reflect gradual improvements in different separations, but on its own is of little value. Two peaks that are well resolved and easy to separate will dominate the sum and optimization may result in this peak pair being over separated, while the most difficult pair to separate is ignored. One way of avoiding this problem is to sum the resolutions but to limit the maximum resolution that can be assigned to any peak pair. Taking the separation time into account the chromatographic optimization function (COF) can be defined as

$$\text{COF} = \sum_{i=1}^n A_i \ln (R_i / R_{id}) + B(t_x - t_n) \quad (1.46)$$

where R_i is the resolution of the i th pair, R_{id} the desired resolution for the i th pair, t_x the maximum acceptable retention time for the last eluted peak, t_n the observed retention time for the last eluted peak, and A_i and B are arbitrary weighting factors used to indicate which peaks are more important to separate than others, and to allow flexibility in setting the acceptable separation time. The COF function gives a single number that tends towards zero as the optimum separation is reached while poor chromatograms produce large, negative values. The COF, however, makes no allowance for peak crossovers and identical values of the COF can result from chromatograms with different numbers of separated peaks. Rather than add the individual resolution values the product of those values can be used. In this case the aim is to space the peaks evenly throughout the chromatogram, since the lowest value of the resolution has a dominant effect. A simple resolution product may still give a higher assessment to an inferior chromatogram but this can be overcome by using the relative resolution product, where the denominator defines the maximum possible value for the resolution product in the given chromatogram. In addition, the relative resolution product can be modified to include a term that incorporates the importance of the separation time in obtaining the desired separation.

None of the functions considered so far specifically takes into account the number of peaks found in the chromatogram. If the object of the separation is to detect the maximum number of peaks, even if the resolution of individual peaks is poor, then Eq. (1.46) and similar equations will be inadequate. A chromatographic response

function, CRF, which takes into account the simultaneous importance of resolution, separation time, and the total number of detectable peaks, can be expressed as follows

$$\text{CRF} = \sum_j R_j + n^a - b(t_x - t_n) - c(t_0 - t_1) \quad (1.47)$$

where n is the number of peaks observed, t_0 the minimum desired retention time for the first detected peak, t_1 the observed retention time for the first detected peak, and a , b and c are adjustable weighting factors to change the emphasis of the various contributions to the CRF. Usually the exponent is chosen so large (e.g., $a = 2$) that the appearance of a new peak raises the criterion significantly. A problem with the CRF and similar multi-term functions is that their numerical values can become dominated by one of the terms in the expression and fail to represent the intended mix of terms.

From the above discussion it should be obvious that the selection of an appropriate objective function is a difficult task. It is highly likely that different objective functions will result in the location of different optimum experimental conditions for the separation. Yet, it is not possible to set hard guidelines for the selection of a particular objective function, which must be chosen by practical experience keeping the goals of the separation in mind.

1.6.3 Peak Capacity

In the separation of complex mixtures the total number of observed peaks is as important or more so than the resolution of specific peak pairs. Many of these peaks may not be singlets (see section 1.6.4), but systems that separate the mixture into the largest number of observable peaks are desirable unless only a few components are of interest. The separating power of a column can be characterized by its peak capacity, defined as the maximum number of peaks that can be separated with a specified resolution in a given time interval, Figure 1.16. For the general case it can be calculated using Eq. (1.48)

$$n_C = 1 + \int_{t_M}^{t_R} (\sqrt{N} / 4t) dt \quad (1.48)$$

where n_C is the peak capacity and t the separation time [210-214]. For simplicity a resolution of 1.0 is usually adopted and it is assumed that all the peaks are Gaussian. However, the integration of Eq. (1.48) is not straightforward. In many chromatographic systems the plate number is not independent of retention time. Ignoring the variation of the plate number with retention time Eq. (1.48) can be integrated to give

$$n_C = 1 + (\sqrt{N} / 4) \ln (t_R / t_M) \quad (1.49)$$

where t_M is the column hold-up time and t_R the maximum retention time for elution of the last peak. Alternatively, the ratio (t_R / t_M) can be replaced by the ratio (V_{\max} / V_{\min}) where V_{\max} and V_{\min} are the largest and smallest volumes, respectively, in which a solute can be eluted and detected. The numerical value of n_C depends on t_R and unless

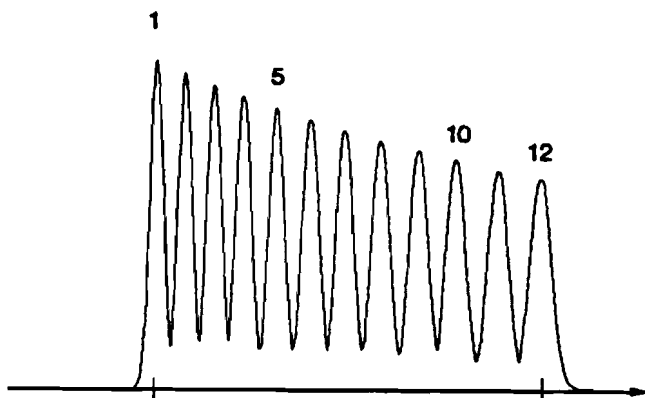


Figure 1.16. Simulation diagram of the column peak capacity.

some method is used to define the useful retention space then unrealistic values for the peak capacity are possible with no connection to chromatographic practice. Using the ratio of (V_{\max} / V_{\min}) is one way to achieve this, although it should be noted that in this form the peak capacity depends on detector characteristics as well as column properties. Eq. (1.49) is a reasonable approximation for packed columns in gas and liquid chromatography but is generally unsuitable for open tubular columns. The relationship between N and the retention factor is more complex for open tubular columns, particularly for small values of the retention factor, for which Eq. (1.49) underestimates n_C . Unfortunately, general solutions to Eq. (1.49) that accommodate small retention factors are complex and may require numerical integration or approximations for evaluation [211]. Similar problems arise for programmed separation conditions, for which the best solution is to express the time variation of peak widths by some empirical experimental function for the whole or segments of the separation [213]. The segment values are then summed to provide the total peak capacity. Alternatively, automated measurements of peak widths throughout a chromatogram enable the peak capacity to be calculated by integration of the curve generated from a plot of reciprocal peak widths against retention time [215]. This method has the advantage of providing a rigorous experimental value for the peak capacity. Martin states that typical peak capacities for isocratic liquid chromatography are between 50-70; for programmed liquid chromatographic separations 200-500; programmed separations by gas chromatography up to 2000-5000; and for capillary electrophoretic techniques 500-5000 [216]. The peak capacity for thin-layer (section 6.3.8) and size-exclusion (section 4.9.3.1) chromatography are discussed elsewhere.

The separation number (or Trennzal) is a special case of the peak capacity. It is widely used as a column quality test in gas chromatography [217-219] and to a limited extent in micellar electrokinetic chromatography [220,221]. It is defined as the number of peaks that can be placed between the peaks of two consecutive homologous standards with z and $z+1$ carbon atoms and separated with a resolution of $R_S = 1.177$. It represents the

integral solution of Eq. (1.49) when the time limits correspond to the retention time of the two homologous standards, usually *n*-alkanes or fatty acid methyl esters in gas chromatography and alkylaryl ketones in micellar electrokinetic chromatography. Since the peak resolution used to define the peak capacity and the separation number (SN) differ the two terms are related by $n_C = 1.18 (SN + 1)$, at least for well retained peaks. The separation number is calculated from the retention time (t_R) and peak widths at half height (w_h) for the homologous standards using Eq. (1.50)

$$SN = ([t_{R(z+1)} - t_{R(z)}] / [w_{h(z)} + w_{h(z+1)}]) - 1 \quad (1.50)$$

The separation number depends on the properties of the stationary phase, the column length, column temperature and carrier gas flow rate. For meaningful column comparisons it is necessary to standardize the separation conditions. The critical parameters for obtaining reproducible SN values in the temperature program mode are the identity of the standards, the carrier gas flow rate and the temperature program rate [218]. Blumberg and Klee [222] introduced the separation measure, *S*, as an indication of the separation capacity for isothermal and temperature-programmed gas chromatography. It is evaluated from a series of relationships that depend on the experimental conditions. It has the useful property of being an additive function but has so far not proven popular.

The confident analysis of moderate to complex mixtures requires a large peak capacity. Multidimensional chromatographic techniques that combine distinctly different separation mechanisms for each component dimension provide a powerful approach to obtaining a large peak capacity [223-225]. The peak capacity for a multidimensional system is approximately multiplicative, while series coupling of identical columns only results in an increase in peak capacity that is roughly equal to the product of the square root of the number of coupled columns and their individual peak capacities. Thus, if two identical columns with a peak capacity of 100 were coupled in series, then the resultant peak capacity would be about 140, compared to a value of about 10,000 for two columns of the same peak capacity used in the multidimensional mode. The multiplicative rule provides only an estimate of the peak capacity in two-dimensional separations since correlation of solute retention in the two dimensions reduces the available retention space. Many two-dimensional separation systems employ retention mechanisms that differ in intensity only, for example, the use of reversed-phase liquid chromatography with two different stationary or mobile phases. Consequently the observed total peak capacity is only a fraction of the product of the two one-dimension peak capacities. Coupling of different separation modes, such as reversed-phase liquid chromatography and capillary electrophoresis or normal-phase chromatography and gas chromatography, are examples of two-dimensional systems that can provide large peak capacities. Although a large peak capacity is required for the separation of complex mixtures the actual number of components that can be isolated in a separation is far less than the theoretical peak capacity due to statistical overlap of component peaks. The statistical theory of peak overlap predicts that the ability to resolve peaks in two-dimensional separations does not increase in direct proportion to the increase in peak capacity [226].

1.6.4 Statistical Overlap Models

The peak capacity provides an overoptimistic assessment of the real resolving power of a chromatographic system. Real samples do not contain peaks that will emerge exactly at the correct retention time to fulfill the condition of unit resolution. The peak capacity concept can be combined with statistical models that assume that the component peaks of complex mixtures distribute themselves randomly, or according to some other selected probability function, along the retention axis and then solved to indicate the extent of peak overlap [183,226-228]. General theories of statistical overlap are of two types. In the first and more common, the interval between adjacent peaks are interpreted using point-process [229-231] or pulse-point [232] statistics. These models differ in that multicomponent chromatograms are processed as consisting of random point positions in the point-process models and of random pulses at random positions in the pulse-point model. The second approach is based on Fourier analysis to determine the frequency content of the separation [227,233,234]. The correct use of these theories requires a distribution model (such as the Poisson function for randomly distributed peaks). A probability function for the resolution required to establish a minimum peak separation (because the range of peak sizes in the mixture and the degree of saturation affect this). Some model or assumptions concerning the distribution of peak widths in the chromatogram (in programmed separations it can be reasonably assumed that all peaks have the same width). Not surprisingly these models are mathematically complex, but reasonably successful for interpreting the complexity of real chromatograms. Roughly summarized, statistical models of peak overlap suggest that there is a reasonable probability of separating mixtures containing up to about 40 components into single peaks by state-of-the-art chromatographic systems provided that these systems have a large peak capacity. For more complex mixtures or systems with low or moderate peak capacities an increasing number of observable peaks will contain different numbers of unresolved components. Statistical overlap models provide an excellent tool to estimate the number of single component peaks and the number and composition of multiplets of different order. At high saturation (ratio of observed peaks to the peak capacity) the number of single component peaks will be small and most observed peaks will be multiplets of different order.

1.7 SEPARATION TIME

In the general theory of chromatography, efficiency and peak capacity are optimized to provide a specified separation in a reasonable time. In certain circumstances it might be more desirable to minimize the separation time and accept some compromise in the separation quality. For example, when a high sample throughput is required or for monitoring changes in fast chemical processes. The separation time is often referred to as the analysis time and time-optimized chromatographic separations as high-speed or fast chromatography [138,154,235,236]. The experimental conditions

for time-optimized chromatography can be derived from a simple three-component model. This model assumes that the optimum column length is dictated by the plate number required to resolve the two most difficult to separate components and the total separation time by the time required for the last peak to elute from the column. At a constant temperature (GC) or solvent composition (LC) Eq. (1.45) defines the plate number required for the separation and the corresponding time is given by Eq. (1.3). After some algebraic manipulation, the time required to separate the critical pair is given by Eq. (1.51)

$$t_R = 16R_S^2 [\alpha / (\alpha - 1)]^2 [(1 + k)^3 / k^2] [H / u] \quad (1.51)$$

When the critical pair is separated, so are all other peaks in the chromatogram. If the critical pair is not the last two peaks in the chromatogram the separation time will be $t_R(1 + nk)$ where n is the ratio of the retention factor of the last peak to elute in the chromatogram to the retention factor of the second peak of the critical pair. The fastest separation is obtained when t_R is minimized. This will be the case if the following criteria are met. The minimum useful value for the resolution of the critical pair is accepted. The separation system is optimized to maximize the separation factor (α) for the critical pair. The retention factor for the critical pair is minimized ($k \approx 1 - 5$). The column is operated at the minimum value of the plate height, H_{\min} , corresponding to u_{opt} .

For gas chromatography the ratio (H / u) is a complex function of the operating conditions (pressure drop, film thickness, column dimensions, mass transfer properties, etc). Comprehensive theory is available for open tubular and packed columns operated under different conditions providing all relevant equations [130,131,237-239]. Extremely fast separations (separations in a few seconds or less) are only possible when the plate number required to separate the critical pair is low, because short columns are needed for these separations. Reduction in the column diameter for open tubular columns or particle size for packed columns is the best general approach to increase the separation speed in gas chromatography. The price paid is an increase in the column pressure drop. Since open tubular columns are more permeable than packed columns they provide faster separations for most operating conditions. The disadvantage of narrow-bore open tubular columns is their low sample capacity, roughly equal to $2000(r_{\text{cdf}})$ where r_c is the column radius (mm) and $_{\text{df}}$ the film thickness (μm) when the sample capacity is given in nanograms. Thin-film, narrow-bore columns have typical sample capacities of 10-20 ng per component and are easily overload by major sample components. Packed columns provide high sample capacities, virtually independent of particle size, but are really only useful for fast separations of very simple mixtures [240,241]. Low viscosity and favorable solute diffusion properties make hydrogen the preferred carrier gas. Hydrogen allows higher flow rates or longer columns to be used with a fixed inlet pressure. Helium and nitrogen are 50% and 250%, respectively, slower than hydrogen. Vacuum-outlet operation, for example in GC-MS (section 9.2.2.1), allows faster separations for short and wide-bore columns but is less influential for long or narrow-bore columns (in

this case the inlet and average column pressure are hardly influenced by the outlet pressure) [242-245]. Vacuum-outlet operation of short and wide-bore columns allows the entire column to operate at a lower pressure shifting the optimum mobile phase velocity to higher values and providing more favorable solute diffusion coefficients.

High-speed separations, particularly with open tubular columns of 100- μm internal diameter or less, place special demands upon instrumentation [246-250]. The gas chromatograph must be capable of high-pressure operation; sample introduction systems must be capable of delivering extremely small injection band widths (few milliseconds); the detector and data acquisition system must have a fast response (response times of a few milliseconds); and the system should have an extremely small extracolumn dead volume. In addition, the program rate has to be increased in proportion to the reduction in column diameter for temperature programmed separations, requiring ovens that allow very high programming rates (e.g., 100°C/min). Standard gas chromatographs were not designed to meet these specifications and it is only in the last few years that suitable instruments for fast gas chromatography became available.

Liquid chromatographic separations will never be as fast as gas chromatographic separations because mass transfer properties in liquids are inferior to those in gases [138,154,235,236,251]. Most fast separations in liquid chromatography are accomplished at the maximum available inlet pressure. Adopting reduced parameters (section 1.5.3) the separation time is given by

$$t_R = h^2 \phi N_{\text{req}}^2 \eta / \Delta P \quad (1.52)$$

The fastest possible separation with a given column pressure drop (ΔP) and N_{req} is obtained at the minimum reduced plate height (h_{min}) corresponding to the optimum reduced mobile phase velocity (v_{opt}). This will be achieved at a specified optimum particle size (d_{popt}) according to

$$d_{\text{popt}} = \sqrt{(h v N_{\text{req}} D_M \eta \phi / \Delta P)} \quad (1.53)$$

Since sorbents are available in a limited number of particle sizes, a compromise is necessary. An available particle size is selected that is larger but closest to d_{popt} . The column is made proportionately longer and operated at a slightly higher velocity than v_{opt} . The retention time, however, will be slightly longer than the theoretical minimum. Elevated temperatures are useful for fast separations because of decreased mobile phase viscosity (η) and increased solute diffusion (D_M).

An alternative strategy for fast liquid chromatography uses short columns packed with small particles operated at high flow rates and often elevated temperatures to separate simple mixtures under conditions where resolution is compromised but still adequate for identification purposes [252-258]. Small diameter particles provide larger plate numbers by virtue of their relatively small interparticle mass transfer resistance combined with a shallow increase in the reduced plate height as the reduced mobile

phase velocity is increased beyond its optimum value (see Figure 1.9). Both features are favorable for fast separations. Low column permeability and the limited inlet pressure dictate that short columns are essential, as well as favorable for fast separations, but total plate numbers are low. These short columns (1 – 5 cm) usually packed with 3- μm particles and operated with fast gradients (1-5 min) have found a niche in the pharmaceutical industry for rapid screening of combinatorial libraries and drug metabolite profiling [257,258]. Mass spectrometric detection is frequently used to track analytes to accommodate poor peak separations. It is the synergy between mass separation and chromatographic separation that provides acceptable identification of analytes with separation times typically between 1 to 10 minutes per sample. These miniaturized separation systems require adapted instrumentation because of the small extracolumn volumes and narrow peak profiles.

The fast separation of macromolecules requires special nonporous or perfusive stationary phases with favorable retention and mass transfer properties [138, 259]. Combined with elevated temperatures, steep gradients, high flow velocities and short columns these stationary phases are very effective for fast separations of biopolymers. Supercritical fluids have more favorable kinetic properties for fast separations than liquids but are restricted to operating conditions where the density drop along the column is minimal to avoid unfavorable increases in solute retention with migration distance [260]. Compared with gas chromatography narrow-bore open tubular columns are a poor compromise for fast separations using supercritical fluids because they yield small retention factors and provide low efficiency per unit time. Packed columns with a specific ratio of length to particle diameter to provide the desired resolution are generally used.

Separation speed in capillary electrophoresis is governed by a different set of dynamics compared to liquid chromatography. Efficiency is independent of the column length but migration time is proportional to the length squared [236,261,262]. It is the voltage applied across the capillary column that determines the plate number. Short columns can provide both high plate numbers and fast separations with the minimum column length established indirectly by extracolumn contributions to band broadening and the capacity of the capillary to dissipate the heat generated by the current passing through the column. The migration time (t_f) is given by $Ll / \mu V$ where V is the voltage applied across the column of length L , l is the distance migrated by the sample from the point of injection to the point of detection (generally less than L when on-column detection is used), and μ is the mobility of the ion. Separations on a millisecond time scale are possible, if far from routine, using very short capillaries and microstructures (section 8.9.7).

1.8 PRINCIPLES OF QUANTIFICATION

This section reviews the basic performance characteristics of chromatographic detectors and the various methods of obtaining quantitative information from their signals.

1.8.1 Signal Characteristics

The fundamental properties of the detector signal of general interest are sensitivity, limit of detection, dynamic and linear ranges, response time and noise characteristics. It is convenient to divide chromatographic detectors into two groups based on their response characteristics. Concentration sensitive detectors are non-destructive and respond to a change in mass per unit volume (g/ml). Mass sensitive detectors are destructive and respond to a change in mass per unit time (g/s). Many liquid phase detectors, such as UV-visible, fluorescence, refractive index, etc., are concentration sensitive detectors, while many of the common gas-phase detectors (e.g. flame-based detectors) are mass sensitive. Sensitivity is defined as the detector response per unit mass or concentration of test substance in the mobile phase and is determined as the slope of the calibration curve for detectors with a linear response mechanism. For a concentration sensitive detector the sensitivity, S , is given by $S = AF / w$ and for a mass sensitive detector by $S = A / w$, where A is the peak area, F the detector flow rate, and w the sample amount. A detector with a high sensitivity, corresponding to a larger slope, is better able to discriminate between small differences in the amount of analyte. Sensitivity is often incorrectly used for limit of detection (or limit of determination). Colloquially a detector with a low limit of detection is sometimes incorrectly referred to as a sensitive detector when inferring that the detector provides a useful response to a small amount of sample. A sensitive detector would be one that is able to distinguish a small range of sample sizes. The limit of detection is defined as the concentration or mass flow of a test substance that gives a detector signal equal to some multiple of the detector noise. A value of 2 or 3 is commonly used as the multiple. The limit of detection (D or LOD) is given by $D = aN / S$, where a is the multiple assumed in the definition of the limit of detection. When the test substance is also specified it can be used to compare the operating characteristics of different detectors under standard conditions.

The detector output contains signal associated with the response of the detector to the analyte and noise originating from the interaction of the detector with its environment and from its electronic circuitry [183,263,264]. There are three characteristic types of noise recognized as short term, long term and drift with properties that can change depending on whether they are determined under static or dynamic conditions. Static noise represents the stability of the detector when isolated from the chromatograph. Dynamic noise pertains to the normal operating conditions of the detector with a flowing mobile phase. Ideally, the static and dynamic noise should be similar, and if not detector performance is being degraded by the other components of the chromatograph. The noise signal is measured over a period of time with the detector set to its maximum usable operating range. The observed noise will be different depending on the recording device because virtually all-normal laboratory recording devices include some form of noise filter. Short-term noise is defined as the maximum amplitude for all random variations of the detector signal of a frequency greater than one cycle per minute, Figure 1.17. It is calculated from the recording device by dividing the detector output into a series of time segments less than one minute in duration and summing the vertical displacement of each segment over a fixed time interval, usually 10 to 15 minutes.

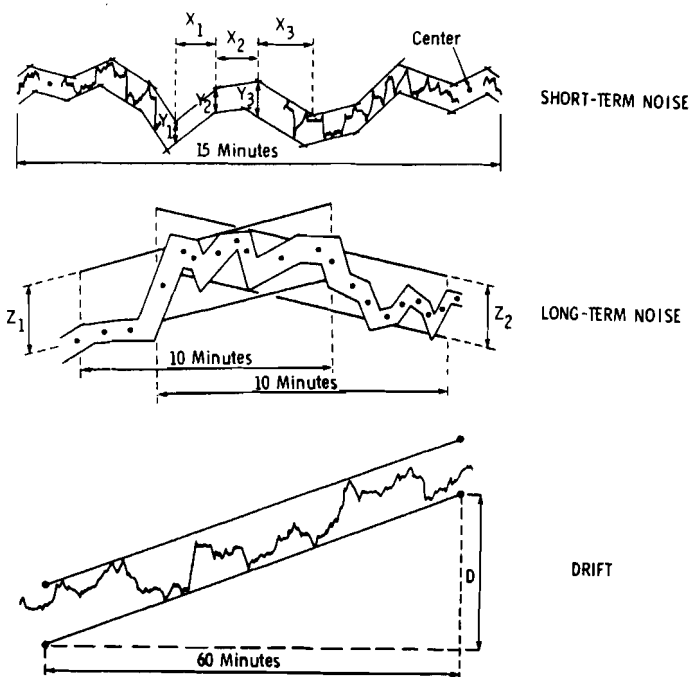


Figure 1.17. Methods for calculating noise for chromatographic detectors.

Long-term noise is the maximum detector response for all random variations of the detector signal of frequencies between 6 and 60 cycles per hour. The long-term noise is represented by the greater of Z_1 and Z_2 in Figure 1.17. The vertical distances Z_1 and Z_2 are obtained by dividing the noise signal into ten-minute segments and constructing parallel lines transecting the center of gravity of the baseline deflections. Long-term noise represents noise that can be mistaken for a late eluting peak. Drift is the average slope of the noise envelope measured as the vertical displacement of the baseline over a period of 1h. For spectrophotometric detectors, the signal response is proportional to the path length of the cell and noise values are normalized to a path length of 1 cm.

The dynamic range of the detector is determined from a plot of detector response or sensitivity against sample amount (mass or concentration). It represents the range of sample amount for which a change in sample size induces a discernible change in the detector signal. For many, although not all, chromatographic detectors the relationship between response and analyte mass or concentration is linear for a wide range of analyte concentration. It is the extent of this range that is of most interest to analysts. The linear range is commonly used for all quantitative determinations. It is the range of sample amount over which the response of the detector is constant to within 5%. It is usually expressed as the ratio of the highest sample amount determined from the linearity plot to the limit of detection for the same compound (Figure 1.18).

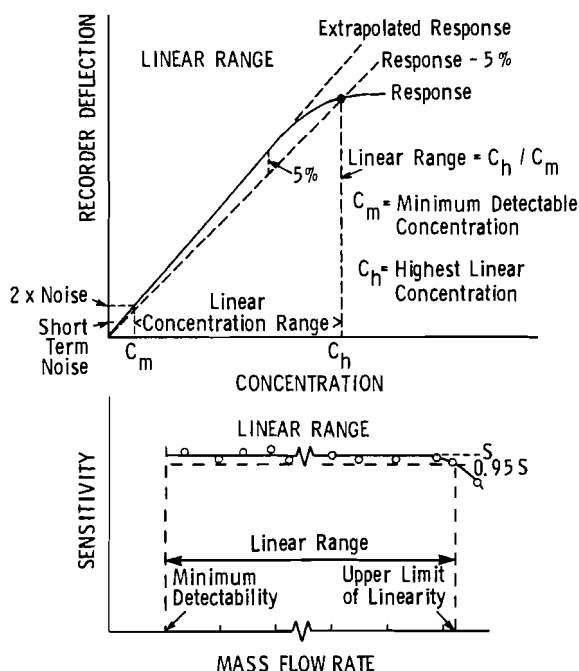


Figure 1.18. Methods for calculating the linear response range for chromatographic detectors.

Table 1.11

Comparison of manual methods for determining peak areas

H = peak height, H' = peak height by construction (see Fig. 1.4) and w_h = peak width at half height, w_b at base, $w_{0.15}$ at 0.15H, $w_{0.25}$ at 0.25H and $w_{0.75}$ at 0.75H

Method	Calculation	True area (%)	Precision (%)
Gaussian peak	Hw_h	93.9	2.5
Triangulation	$H'w_b/2$	96.8	4.0
Condol-Bosch	$H(w_{0.15} + w_{0.75})/2$	100.4	2.0
EMG	$0.753Hw_{0.25}$	100	2.0
Planimetry		100	4.0
Cut and weigh		100	1.7

1.8.2 Integration Methods

For quantitative analysis it is necessary to establish a relationship between the magnitude of the detector signal and sample amount. The detector signal is measured by the peak area or height from the chromatogram. A number of manual methods are available for calculating peak areas, Table 1.11 [3,184,264,265]. For Gaussian peaks the product of the peak height and the width at half height or the method of triangulation can be used. In both cases the calculated area is less than the true peak area. This is only important if the absolute peak area is used in further calculations. In this case

the calculated area should be multiplied by the appropriate factor to give the true peak area. When peak area ratios are used, as in calibration, this is not important. The Condol-Bosch and exponentially modified Gaussian (EMG) models provide a reasonable estimate of the peak area for Gaussian and moderately asymmetric peaks. Up to an asymmetry factor of 3 in the case of the EMG method. No single method is perfect and common problems include the difficulty of accurately defining peak boundaries, variable precision between analysts and the need for a finite time to make each measurement. A major disadvantage of manual measurements is the necessity that all peaks of interest must be completely contained on the chart paper (or adjusted to remain on the chart paper by varying the detector attenuation during the separation). This severely limits the dynamic range of solute composition that can be analyzed. For those methods that depend on the measurement of peak widths narrow peaks are usually difficult to measure with acceptable accuracy using a magnifying reticule or comparator unless fast chart speeds are used to increase the peak dimensions.

Planimetry and cutting out and weighing of peaks require no assumptions about the shape of the peak profile and can be used to determine the area of asymmetric peaks. The proper use of a planimeter (a mechanical device designed to measure the area of any closed plane by tracing out the periphery of the plane with a pointer connected by an armature to a counter) requires considerable skill in its use. Even so, obtaining accurate results requires repetitive tracing of each peak with the totals averaged. The cut and weigh procedure depends critically on the accuracy of the cutting operation. The homogeneity, moisture content and weight of the paper influence precision. Copying the chromatogram onto heavy bond paper, with expansion if possible, will preserve the original chromatographic record of the separation and enhance the precision of the weighings.

Dedicated electronic integrators and personal computers with appropriate software for integration are routinely used for recording chromatograms [183,184,264,266-271]. Since manual methods for determining peak information are tedious and slow, this is hardly surprising. It is important, however, to understand the limitations of electronic integration. Computer-based systems are increasingly used since they can combine instrument control functions with chromatogram recording and integration as well as providing electronic data storage. Computer-based systems also provide flexible approaches for reporting results and for performing advanced data analysis techniques using additional resident software.

The continuous voltage output from chromatographic detectors is not a suitable signal for computer processing. The conversion of the detector signal to a computer readable form requires an interface usually resident as an expansion card in the computer. The interface scales the detector output to an appropriate range, digitizes the signal, and then transfers the data to a known location in the computer. The original input signal is transformed into a series of voltages on a binary counter and is stored as a series of binary words suitable for data processing.

The important characteristics of the analog to digital conversion device are its sampling frequency, resolution and range. The accurate recording of chromatographic

peaks requires that at least 10 data points are collected over the peak width. The sampling frequency must match the requirements for the narrowest peak in the chromatogram. For modern devices with sampling frequencies of 5 Hz or better undersampling is usually only a problem in high-speed chromatography [250,268]. Analog to digital converters used for chromatographic applications are usually auto-ranging, meaning that the detector signal is automatically divided into ranges so as to provide sufficient resolution near the baseline for peak detection while accurately registering the peak maximum for large signals. The resolution of the analog to digital converter is the smallest change in the analog signal that can be seen in the digital output (specified as the number of bits).

Converted data is usually averaged (bunched) to minimize storage space. Using the local peak width to determine the frequency of bunching creates uniform sampling density throughout the chromatogram. Long stretches of stable baseline are stored in one bunch represented by a single datum and the number of times it recurs. Stored data is initially smoothed and peak locations identified by a slope sensitivity function. Small peaks and baseline artifacts are removed using a threshold function or later by a minimum area reject function. Standard procedures may fail in the case of complex baselines, tailing peaks or excessive peak overlap. Computer-based integration software often tries to compensate for this problem by providing the possibility of video integration. The operator can set any integration boundaries desired by moving a cursor on the video monitor. Choosing boundaries though is arbitrary, and it is quite likely that different operators will choose different boundaries. Because this procedure can not be validated it is unsuitable for regulatory and general quality assurance problems. It can be useful in other circumstances to correct blunders made by the software in logically interpreting the correct boundary positions.

Most often, the derivatives of the smoothed signal are used for peak detection [183,184,270-275]. Peaks are detected because the detector signal amplitude changes more rapidly when peaks elute than the baseline signal does between peaks. There is a threshold of slope below which peaks are not detected as different to baseline fluctuations, and this value is set by the slope sensitivity factor. Differentiation of the detector signal enhances the changes within the signal facilitating the accurate location of peak start and end positions. The starting point of the peak is detected when the first derivative has reached a predetermined threshold value. The peak maximum and also the valley between partially resolved peaks are then indicated where the derivative falls to zero and the end of the peak where the derivative drops below the threshold and the signal returns to the baseline. In order to avoid the detection of false peaks due to abrupt changes caused by baseline noise a minimum peak width is usually predefined and the detected peak is accepted only if the difference between the start and end of the peak exceeds the threshold. Some systems use a two step peak-indicating algorithm. In the first step a coarse estimation of peak positions is made when the first derivative exceeds the threshold for two or more consecutive data points. Then the second derivative of the signal is analyzed backwards and peak positions reassigned with respect to the chosen threshold in the usual way. The choice of threshold value is very important

and is often user selectable. Alternatively, the integrator may use the average value of the signal fluctuations at the start of the chromatogram to automatically self-program an appropriate threshold value. With a low threshold, noise cannot be distinguished from peaks, and too many signals will be reported. With too high a threshold, small peaks are overlooked and there will be a late start to the integration of detected peaks.

The area between the start and end positions identified for the peak is integrated using a summation algorithm usually based on Simpson's rule or trapezoidal integration. Simpson's rule is more accurate because it fits a quadratic function to groups of three consecutive data points whereas the trapezoid method involves fitting a straight line between data points. An adequate sampling frequency is also important since this determines the number of slices across the peak (i.e. the data points that are integrated). Subtracting the baseline area from the accumulated integral count completes the peak area calculation.

In the absence of significant baseline noise most integrators should be capable of high precision and accuracy when integrating isolated symmetrical peaks. Examples of peaks often poorly treated by computing integrators are small peaks with large peak widths, peaks on the tail of larger peaks or the solvent front, peaks on a noisy baseline and fused peaks [183,184,268,276,277]. Noise blurs the determination of base peak widths by making it difficult to locate the exact peak start and end positions. Noise at the peak tops can cause integrators to split area measurements when valley recognition is triggered by the micro-peaks. Ultimately, noise determines the smallest peak size that can be distinguished from random baseline fluctuations.

Most computing integrators construct baselines as a straight line drawn beneath the peak. Real chromatograms with a rising baseline may have a more complex baseline structure resulting in both gains and losses in the true peak height and area depending on the shape of the real baseline, Figure 1.19. Fused peaks are generally integrated by dropping perpendiculars from the valley between them or, if one peak is much smaller than the other and located on its tail, by skimming a tangent below it, Figure 1.19. The measurement errors introduced by perpendicular separation of two peaks are determined by their relative sizes, their asymmetries, the elution order and degree of overlap [184,270,277-279]. If peaks are symmetrical but unequal in size, perpendicular separation over-estimates the smaller area at the expense of the larger one. If the peaks are asymmetric there is a larger inequality in the contribution of each peak to the other. Tangent skimming under-estimates the area of the small peak unless it is very much smaller and narrower than the peak from which it is skimmed. The general conclusion is that there is no secure method for the accurate integration of fused peaks.

Area is the *de facto* peak characteristic related to the mass or concentration of analyte detected [184,270,278-283]. Height is susceptible to peak asymmetry and retention differences while area is not, and the linear dynamic range for area measurements is greater than for height. Under some circumstances peak height might provide a more reliable or reproducible estimate of the analyte present. When the signal to noise ratio is small, height is preferred because errors of baseline placement affect height less than area. For manual measurements of well-separated and symmetrical peaks

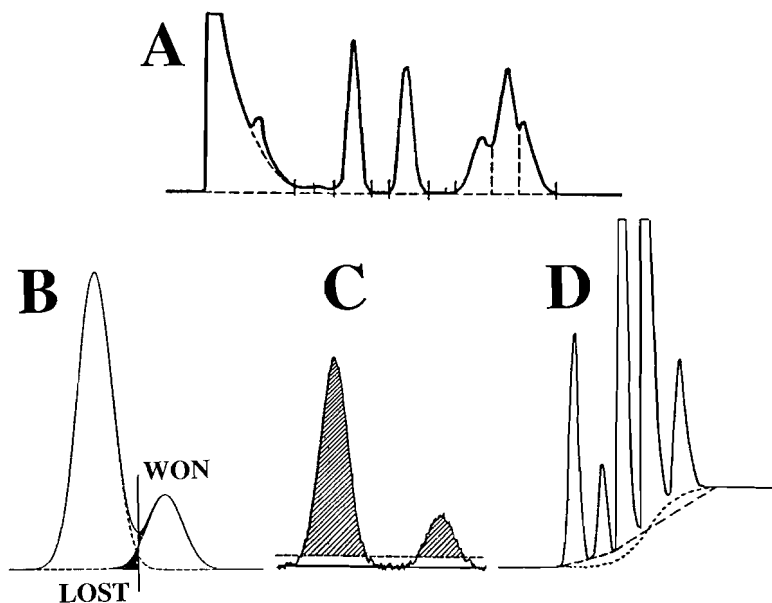


Figure 1.19. Possible error sources in the integration of chromatographic peaks. (A) Tangent skimming and perpendicular drop methods to assign peak boundaries for fused peaks; (B) the misproportionation of the true peak area between two incompletely separated peaks of different size; (C) loss of peak area due to integration with a threshold value set too high; and (D) variation of peak area due to differences between the real sloping baseline and the interpolated integrator baseline.

height is less tedious to determine than area. In other circumstances the precision and accuracy of peak height and area measurements depend on several chromatographic variables, including sample size, mobile phase composition, flow rate, and column temperature. When a mass-sensitive detector is used, quantification should be based on peak area since precision of the measurement will be independent of flow rate, temperature stability and other chromatographic factors that alter either the elution volume or peak shape. In contrast, when a concentration-sensitive detector is used the peak height should be independent of flow rate variations and could be more precise than area measurements. For liquid chromatography area measurements are preferred when the column flow rate can be controlled precisely even if the mobile phase composition shows some variability and *vice versa* as far as peak height measurements are concerned. Quantification in gradient elution chromatography requires careful control of total flow rate when peak areas are used and gradient composition when peak heights are used. To test which alternative is applicable, variation in the retention time of early eluting peaks indicates poor flow precision and variation in the retention time of late eluting peaks suggests poor precision in the mobile phase composition. Consequently, whether peak height or peak area is selected for a particular analysis depends on system performance and not necessarily on sample composition. For fused

peaks, peak height is sometimes more accurate for tailing peaks of equal area as well as for the smaller of two peaks when the small peak is eluted first and the area ratio is large and for the smaller of two peaks when the small peak is eluted last if the peaks are symmetrical.

1.8.3 Relative Composition

Four techniques are commonly used to convert peak heights or areas into relative composition data for the sample. These are the normalization method, the external standard method, the internal standard method and the method of standard additions [264,284]. In the normalization method the area of all peaks in the chromatogram are summed and then each analyte is expressed as a percentage of the summed areas. All sample components must elute from the column and their responses must fall within the linear operating range of the detector. This method will always lead to totals representing 100%. If the detector response is not the same for all compounds then response factors are required to adjust the peak areas to a common scale. Response factors are usually determined as the slope of the calibration curve and converted to relative response factors since these tend to be more stable than absolute values.

The external standard method compares peak areas in an unknown sample with the areas produced by a known amount of the same analytes in a standard sample analyzed under identical conditions. Standards are chromatographed separately alternating in order with samples for the highest precision. The standard will normally be a synthetic mixture with individual components at a similar concentration to those of the sample. No response factors are required, therefore, and peak shape and linearity problems are avoided. The standard solutions should be prepared in the same matrix as the sample. High precision requires absolute constancy of the separation conditions. Equal size injections are essential and injection accuracy and precision are critical parameters. Chromatographic conditions must be stable and all measurements made within the linear operating range of the detector. External standards are frequently employed in quality control applications of raw materials, drugs and formulations, etc., where mostly the major components are analyzed and strict requirements on accuracy and precision of the method apply (0.5 to 2.0% RSD). Note that if the standard and sample components are not identical or one standard is used to determine more than one component, then the appropriate response factors must be determined as described for the normalization method. Using the deferred standard method can minimize the separation time for the external standard method. Injection of the standard is delayed until some time after the sample injection so that it elutes in a region of the chromatogram free of other components.

There are two distinct uses of an internal standard. It can be a substance or substances added to the sample prior to injection to allow absolute quantities of analytes to be measured by compensating for variation in the injected sample volume. Alternatively, it can be a substance or substances that are added to the sample at the earliest possible point in an analytical scheme to compensate for sample losses occurring during

sample preparation and chromatographic analysis. A suitable internal standard must be available in a pure form, have good storage properties and be unreactive towards the sample and its matrix. Since it is added to the sample it cannot be a normal component of the sample and must be completely separated from all other components of the sample. Since it is important that it behaves like the sample components it must have similar physical and chemical properties to them and preferably it should have a similar detector response factor and be present in a similar amount. These conditions are rarely, if ever, met in full, particularly when a single standard is used for the analysis of a multicomponent mixture with sample components varying in relative composition and chemical type. Substances commonly used as internal standards include analogs, homologs, isomers, enantiomers (if a chiral separation system is used), stable isotopically labeled compounds (if a mass detector is used) and radioactive isotopically labeled compounds (if particle detector is used). Analogs and homologs are perhaps the most widely used substances simply because they are likely to be available. Stable isotopically labeled internal standards are frequently used in gas chromatography-mass spectrometry where the mass discriminating power of the mass spectrometer can be used to differentiate between the analyte and internal standard.

When using the internal standard method calibration curves are first prepared for all analytes to be determined and the internal standard to establish the relevant response factors. A constant amount of standard is added to each sample, preferably at a concentration similar to the analytes of interest, and a calibration curve constructed from the ratio of the detector response to the analyte divided by the response to the internal standard plotted against the concentration (amount) of analyte. The ratio of the detector response to the sample unknowns and internal standard is then used for all quantitative measurements. A general problem in the calibration method arises if a least squares fit is used. The error distribution may be no longer normally distributed and when low precision is obtained for the internal standard biased values for the estimated sample concentrations are possible [285]. Poor precision due to variation of the injection volume can largely be eliminated by use of an internal standard. This is frequently a significant error in gas chromatography using manual injection.

The choice of an internal standard for an analytical procedure is often made in a too cavalier a fashion and may actually provide lower precision than external calibration. The successful use of an internal standard depends on the existence of a high correlation between the peak areas (heights) of the analytes and the internal standard for the complete analytical procedure and their being lower variability in the internal standard area (height) compared to those of the analytes [286-288]. An external standard will generally provide higher precision than an internal standard when the variation of the recovery for analytes and standards is sufficiently different and the standard deviation in the mean for repeated analyses of the internal standard is larger than that for the analytes of interest.

The method of standard additions is the least used method of quantitation in chromatography because it requires several repetitive separations to yield a single result [284,289-291]. Equal volumes of the sample solution are taken, all but one are spiked

with known and different amounts of the analyte, and all are then diluted to the same volume. Each sample is analyzed individually and a pseudocalibration curve produced using the known amounts of analyte as the concentration (weight) axis. Extrapolation of the pseudocalibration curve to zero detector response provides an accurate value for the unknown analyte concentration (amount). A lack of linearity in the pseudocalibration plot is a good indication of matrix effects. Analytes and standards must be identical, all solutions analyzed must fall within the linear range of the detector response, absolute constancy of analytical conditions is required (same as for the external standard method), and injection volumes should be identical. Data may be treated statistically by linear regression techniques if a sufficient number of measurements are made.

1.9 REFERENCES

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2.1 INTRODUCTION

Separations are possible in gas chromatography if the solutes differ in their vapor pressure and/or intensity of solute-stationary phase interactions. As a minimum requirement the sample, or some convenient derivative of it, must be thermally stable at the temperature required for vaporization. The fundamental limit for sample suitability is established by the thermal stability of the sample and system suitability by the thermal stability of column materials. In contemporary practice an upper temperature limit of about 400°C and a sample molecular weight less than 1000 is indicated, although higher temperatures have been used and higher molecular weight samples have been separated in a few instances.

When a solid adsorbent serves as the stationary phase the separation technique is called gas-solid chromatography (GSC). When the stationary phase is a liquid spread on an inert support or coated as a thin film onto the wall of a capillary column the separation technique is called gas-liquid chromatography (GLC). In packed column gas chromatography the separation medium is a coarse powder (coated with a liquid for GLC) through which the carrier gas flows. If the adsorbent, liquid phase, or both, are coated onto the wall of a narrow bore column of capillary dimensions, the technique is called wall-coated open tubular (WCOT), support-coated open tubular (SCOT), or porous-layer open tubular (PLOT) column gas chromatography. As indicated by name the characteristic feature of open-tubular columns is their high permeability created by the open passageway through the center of the column.

Five column types have been used in gas chromatography. Classical packed columns have internal diameters greater than 2 mm and are packed with particles in the range 100 to 250 μm . Micropacked columns have diameters less than 1 mm and a similar packing density to classical packed columns (d_p / d_c less than 0.3, where d_p is the average particle diameter and d_c the column diameter). Packed capillary columns have a column diameter less than 0.6 mm and are packed with particles of 5-20 μm diameter. SCOT

Table 2.1

Representative properties of different column types for gas chromatography

 H_{\min} = minimum plate height at the optimum mobile phase velocity u_{opt}

Column type	Phase ratio	H_{\min} (mm)	u_{opt} (cm/s)	Permeability ($10^7 \cdot \text{cm}^2$)
Classical Packed	4-200	0.5-2	5-15	1-50
Micropacked	50-200	0.02-1	5-10	1-100
Packed Capillary	10-300	0.05-2	5-25	5-50
SCOT	20-300	0.5-1	10-100	200-1000
WCOT	15-500	0.03-0.8	10-100	300-20000

columns are capillary columns containing a liquid phase coated on a surface covered with a layer of solid support material, leaving an open passageway through the center of the column. (A PLOT column is simply a SCOT column without the liquid phase). In WCOT columns the liquid phase is coated directly on the smooth or chemically etched column wall. Some characteristic properties of the different column types are summarized in Table 2.1 [1]. The most significant difference among the various column types is their permeability. The open tubular columns offer much lower flow resistance and can be used in much longer lengths to obtain very high total plate numbers. The minimum plate height of the best packed column in gas chromatography is about 2-3 particle diameters whereas that of an open tubular column will be similar to the column diameter. The intrinsic efficiency of open tubular columns, therefore, is not necessarily greater than that of a packed column, but because of their greater permeability at a fixed column pressure drop a greater total plate number can be obtained, since longer columns can be used.

The phase ratio (ratio of the volume of gas to liquid phase) for a number of typical column configurations is given in Table 2.2 [2]. At a constant temperature the partition coefficient will be the same for all columns prepared from the same stationary phase. Consequently, for a column with a large phase ratio the retention factor will be small and *vice versa*. The plate number required for a separation becomes very large at small retention factor values (see Table 1.10). Columns with low phase ratios, that is thick-film columns, have a lower intrinsic efficiency than thin-film columns but provide better resolution of low boiling compounds, because they provide more favorable retention factors. They also allow the separation to be performed in a higher and more convenient temperature range than is possible with thin-film columns. The opposite arguments apply to high boiling compounds that have long separation times on thick-film columns because their retention factors are too large. Increasing the phase ratio lowers the retention factors to a value within the optimum range so that there is little deterioration in resolution and a substantial saving in separation time. Packed columns have low phase ratios compared to WCOT columns leading to longer separation times at a constant temperature. SCOT columns occupy an intermediate position. Since several combinations of film thickness and column radius can be used to generate the same phase ratio, there are other factors that need to be considered for selecting these variables for a particular separation.

Table 2.2

Characteristic properties of some representative columns

 H_{\min} is the minimum plate height and k is for undecane at 130°C

Column type	Length (m)	Internal diameter (mm)	Film thickness (μm)	Phase ratio	Retention factor (k)	H_{\min} (mm)	Column plate number	Plates per meter
Classical	2	2.16	10%(w/w)	12	10.4	0.55	3,640	1,820
packed	2	2.16	5%(w/w)	26	4.8	0.50	4,000	2,000
SCOT	15	0.50		20	6.2	0.95	15,790	1,050
	15	0.50		65	1.9	0.55	27,270	1,820
WCOT	30	0.10	0.10	249	0.5	0.06	480,000	16,000
	30	0.10	0.25	99	1.3	0.08	368,550	12,285
	30	0.25	0.25	249	0.5	0.16	192,000	6,400
	30	0.32	0.32	249	0.5	0.20	150,000	5,000
	30	0.32	0.50	159	0.8	0.23	131,330	4,380
	30	0.32	1.00	79	1.6	0.29	102,080	3,400
	30	0.32	5.00	15	8.3	0.44	68,970	2,300
	30	0.53	1.00	132	0.9	0.43	70,420	2,340
	30	0.53	5.00	26	4.8	0.68	43,940	1,470

In general, increasing the column radius and film thickness for a WCOT column will lead to an increase in the column plate height and decrease in efficiency. However, the relationship between the variables is complex (see section 1.5.2) and depends on the retention factor since this term appears explicitly in the contribution from resistance to mass transfer to the plate height. For thin-film columns ($d_f < 0.25 \mu\text{m}$) resistance to mass transfer in the stationary phase is small (frequently negligible) so that decreasing the radius of thin-film columns by minimizing the mobile phase mass transfer term leads to increased efficiency. Narrow-bore, thin-film columns are the most intrinsically efficient provided that the inlet pressure is not limited. If the column radius is held constant and the film thickness is increased the column efficiency will decline because resistance to mass transfer in the stationary phase will eventually dominate the plate height equation.

Today, WCOT columns and classical packed columns dominate the practice of gas-liquid chromatography and PLOT columns and classical packed columns the practice of gas-solid chromatography. WCOT and PLOT columns are the first choice for analytical separations because of their superior peak capacity and, in the case of WCOT columns, greater chemical inertness. Packed columns are less expensive, require little training in their use, are better suited to isolating preparative-scale quantities, and can better tolerate samples containing involatile or thermally labile components. Only a limited number of stationary phases have been immobilized successfully in open tubular columns compared to the large number of phases available for use in packed columns. Packed columns are still used in many fundamental studies of solvent properties because of the ease of column preparation and the large amounts of solvent that can be accommodated to minimize measurement errors. The attendant difficulties of high-pressure operation and in uniformly packing long columns have conspired to

make micropacked columns the least popular choice for analytical applications [3,4]. Micropacked columns have been used in monitoring extraterrestrial atmospheres because of their favorable combination of durability, high sample capacity and low mobile phase flow rates [5,6]. SCOT columns have more or less been totally replaced by thick-film WCOT columns. They might still prove useful for applications that demand a large sample capacity or use of selective stationary phases that are difficult to coat on open tubular columns or for separations that require a greater plate number than can be provided by classical packed columns. Slurry packed capillary columns with small diameter particles (5-20 μm) have been used for fast separations in instruments modified for high-pressure operation [7-9]. These columns provide higher efficiency per unit time, greater retention and a higher sample capacity than WCOT columns but their low permeability limits the useful column length. Consequently, the total plate number that can be achieved is small ($< 20,000$). They may prove useful for fast separations of simple mixtures but this has to be balanced against the inconvenience of high-pressure operation.

2.2 MOBILE PHASES

It is convenient to divide mobile phases for gas chromatography into solvating and non-solvating gases. The latter are the most important and dominate the practice of gas chromatography. Common carrier gases are hydrogen, helium and nitrogen. These gases behave almost ideally at the low pressures and typical temperatures used in gas chromatography. Consequently, they do not influence selectivity in gas-liquid chromatography except for minor contributions attributed to nonideal gas behavior at extreme operating conditions. In gas-solid chromatography competition between solute molecules and carrier gas molecules for sorbent surface active sites can result in significant changes in retention, particularly for heavier gases like carbon dioxide [10,11]. In this case, retention factors also depend on the average gas pressure for the column. In gas-liquid chromatography the primary function of the carrier gas is to provide the transport mechanism for the sample through the column. The choice of carrier gas may still influence resolution, however, through its effect on column efficiency, which arises from differences in solute diffusion rates. It can also affect separation time, because the optimum carrier gas velocity decreases with the decrease in solute diffusivity. In pressure-limiting conditions, gas viscosity differences are important as well. Other considerations for choosing a particular carrier gas might be cost, purity, safety, reactivity and detector compatibility. These factors also tend to favor the inert gases, nitrogen, hydrogen and carbon dioxide.

The viscosity of the carrier gas determines the column pressure drop for a given velocity. For hydrogen, helium, and nitrogen the viscosity can be calculated for conditions germane to gas chromatography by Eq. (2.1) [12,13].

$$\eta_t = \eta_o(T_t / T_o)^n \quad (2.1)$$

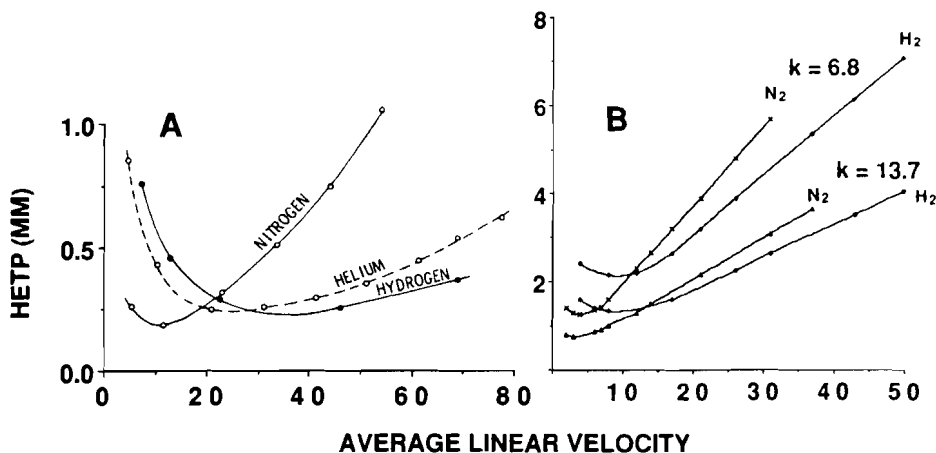


Figure 2.1. Van Deemter plots indicating the influence of the choice of carrier gas on column efficiency for thin-film (A) and thick-film (B) open tubular columns for solutes with different retention factors.

where η_t is the gas viscosity in $\mu\text{P.s}$ ($=\mu\text{N.s/m}^2$) at temperature t , η_0 the gas viscosity at 0°C , and T (K) the temperature. The reference values for the three gases are hydrogen ($\eta_0 = 8.3687$ and $n = 0.6961$), helium ($\eta_0 = 18.6804$ and $n = 0.6977$) and nitrogen ($\eta_0 = 16.821$ and $n = 0.7167$). Tabulated values of gas viscosity at 2°C intervals for the temperature range 0 to 400°C are given in [13]. Differences between the viscosity calculated by Eq. (2.1) and the more exact (although complex) equations derived from the kinetic theory of gases are $< 1\%$ for the temperature range indicated. For the three gases, corrections for pressure differences are negligible up to an inlet pressure of at least 5 atm. Viscosity increases with temperature for gases, causing a decrease in the carrier gas velocity at constant pressure. The rate of change of viscosity with temperature is approximately the same for the three gases. Fused silica, commonly used in the preparation of open tubular columns, is significantly more permeable to helium than either hydrogen or nitrogen [14]. For most circumstances this is unimportant, but for long columns at high temperatures significant loss of helium through the column wall is observed. Column hold-up times can be up to 15% less than predicted by theory but typical deviations are usually only a few percent.

Solute diffusivity influences both the plate height and the position of the optimum carrier gas velocity described by the van Deemter equation [15-18]. Nitrogen provides the lowest plate height, Figure 2.1, but it occurs at an optimum velocity, which is rather low leading to long separation times. In the optimum plate height region the ascending portions of the curve are much shallower for hydrogen and helium. Thus for separations at mobile phase velocities higher than the optimum velocity, compromise conditions selected to minimize separation times, hydrogen, and to a lesser extent helium, show only a modest sacrifice in column efficiency compared to nitrogen.

The above observations are sound for thin-film open tubular columns ($d_f < 0.5 \mu\text{m}$) and low loaded packed columns. Hydrogen is clearly the preferred carrier gas when efficiency and separation time are considered together. For thick-film columns different conclusions are reached, since diffusion in the stationary phase contributes significantly to the band broadening mechanism. In this case the retention factor, temperature, and stationary phase diffusion coefficient have to be considered when selecting a carrier gas for a particular separation [19]. In going from a thin-film to a thick-film column (which corresponds to reducing the phase ratio) the efficiency of the columns, as measured by the minimum plate height, decreases substantially and depends on the carrier gas identity. The optimum carrier gas velocity is reduced by perhaps a factor of two and also depends on the carrier gas identity. The slopes of the ascending portion of the van Deemter curves at high velocity are steeper for the thick-film columns and depend primarily on the stationary phase diffusion coefficient (Figure 2.1). For maximum efficiency thick-film columns should be operated at their optimum mobile phase velocity with nitrogen as the carrier gas. These conditions will enhance resolution while compromising separation time.

For packed columns, resistance to mass transfer in the stationary phase is always significant at normal phase loadings. For pressure limited conditions hydrogen is preferred, because its viscosity is only about half that of helium and nitrogen. Nitrogen provides (slightly) higher efficiency at low temperatures and low flow rates, while hydrogen is superior at higher temperatures and at above optimum flow rates [15,20].

The only disadvantage to the use of hydrogen as a carrier gas is the real or perceived explosion hazard from leaks within the column oven. Experience has shown that the conditions required for a catastrophic explosion may never be achieved in practice with forced air convection ovens. However, commercially available gas sensors will automatically switch off the column oven and carrier gas flow at air-hydrogen mixtures well below the explosion threshold limit. A considerable difference in the relative cost of helium in the USA and Europe has resulted in different preferences on the two continents. For open tubular columns helium is widely used in the USA for safety rather than theoretical considerations while hydrogen is commonly used in Europe.

2.2.1 Solvating Mobile Phases

Occasionally organic vapors or other gases are added to one of the common carrier gases to modify the column separation characteristics [21-25]. Typical examples are steam, ammonia, nitrous oxide, carbon dioxide and formic acid. In general, these gases and their mixtures do not behave ideally and they can influence separations through the solvating capacity of the mobile phase. This is unlikely their main mode of action, however, and changes in peak shape and relative retention caused by masking the adsorption of polar solutes and formation of binary liquid phases through their solubility in the stationary phase are probably more important. For these system selectivity can often be adjusted by changing the partial pressure of the organic vapor in the carrier gas and by varying the column average pressure. In general, however, the inconvenient

experimental arrangement, detector incompatibility and limited temperature range for effective application, combined with the possibility of achieving the desired separations by other conventional means have limited interest in this approach. A more radical approach is represented by solvating gas chromatography, which employs a transition from supercritical fluid to gas phase conditions along the column to modify selectivity (section 7.7.3).

2.3 STATIONARY PHASES

Given the non-solvating properties of the carrier gases commonly used in gas-liquid chromatography optimization of selectivity requires selection of an appropriate stationary phase. Over the history of gas-liquid chromatography thousands of substances have been used as stationary phases [26-30]. For several reasons most of these have been abandoned. Many liquids were technical products of variable or undefined composition unable to meet increasing demands for retention reproducibility. Improved methods of classification indicated that many liquids had similar separation properties and after grouping into classes a few liquids characteristic of each class could suffice for method development. Other liquids had poor thermal stability or high vapor pressure and were replaced by materials with more suitable physical and similar chromatographic properties. Of great significance was the gradual evolution of column technology resulting in increased use of open tubular columns and the preferential selection of liquids that could form stable films on the walls of these columns. This required rather specific properties that many liquids suitable for packed column gas chromatography could not provide. It would not serve our purpose to comprehensively review all available material since much could be arguably identified as redundant. If only the most recent advances were described, though, the reader would be left with no basis to interpret early studies in the field that are still important in piloting the development of column technology. We will attempt to find a middle ground reflecting current practice and a flavor of the recent past.

General considerations that influence the choice of a particular liquid for use as a stationary phase in gas-liquid chromatography include the following. Practical considerations dictate that the liquid should be unreactive, of low vapor pressure, have good coating characteristics (wet the materials used in column fabrication) and have reasonable solubility in some common volatile organic solvent. It is desirable that the liquid phase has a wide temperature operating range covering as far as possible the full temperature range (-60°C to 400°C) used in gas-liquid chromatography. The lower operating temperature for a liquid phase is usually its melting point, or glass transition temperature if a polymer. The lowest temperature at which the mass transfer properties of the melted solid provide adequate column efficiency is an alternative specification for the lowest useful operating temperature. The maximum allowable operating temperature is usually determined by the thermal stability of the stationary phase or its vapor pressure. In practice, the highest temperature that can be maintained

Table 2.3

Characteristic properties of some liquid phases used in packed column gas chromatography

PPE-5 = Poly(phenyl ether), EGS = poly(ethylene glycol succinate) and DEGS = poly(diethylene glycol succinate), Carbowax 20M = poly(ethylene glycol) and FFAP = Carbowax 20M treated with 2-nitroterephthalic acid

Name and structure		Temperature range (°C)	
		Minimum	Maximum
Hexadecane	$C_{16}H_{34}$	< 20	50
Squalane	2,6,10,15,19,23-hexamethyltetracosane	< 20	120
Apolane-87	$(C_{18}H_{37})_2CH(CH_2)_4C(C_2H_5)_2(CH_2)_4CH(C_{18}H_{37})_2$	30	280
Fomblin YR	$-[OCF(CF_3)CF_2]_n(OCF_2)_m-$	30	< 255
PPE-5	$C_6H_5O(C_6H_5O)_3C_6H_5$	20	200
Diethyl Phthalate	$C_6H_4(COOC_2H_5)_2$	< 20	160
EGS	$HO(CH_2)_2[OOCCH_2CH_2COO(CH_2)_2]_nOH$	100	210
DEGS	$HO(CH_2)_2O(CH_2)_2[OOCCH_2CH_2COO(CH_2)_2O(CH_2)_2]_nOH$	20	200
Carbowax 20M	$HO(CH_2CH_2O)_nCH_2CH_2OH$	60	225
FFAP		50	250
1,2,3-Tris(2-cyanoethoxy)propane	$(CH_2OCH_2CH_2CN)_3$	20	170
Tetrabutylammonium Perfluorooctanesulfonate		< 20	220
Tetrabutylammonium 4-Toluenesulfonate		55	200
Tetrabutylammonium Tetrafluoroborate		162	290
Ethylammonium 4-Toluenesulfonate		121	220
Tetrabutylphosphonium chloride		83	230

without breakup of the liquid film into droplets may be used. The liquid temperature range requirement generally favors the selection of polymeric materials, although the chemical structure and composition of polymeric phases is likely to show greater variability than a synthetic compound with a defined chemical structure.

2.3.1 Hydrocarbon and Perfluorocarbon Stationary Phases

High molecular weight hydrocarbons such as hexadecane, squalane ($C_{30}H_{62}$), Apolane-87 ($C_{87}H_{176}$) and Apiezon greases have long been used as nonpolar stationary phases in gas chromatography, Table 2.3. Squalane is obtained by the complete hydrogenation of squalene isolated from shark liver oil. As a natural product its chemical purity is sometimes doubtful with squalene and batyl alcohol being the principal impurities [31]. The Apiezon greases are prepared by the high temperature treatment and molecular distillation of lubricating oils. They are of ill-defined composition and contain unsaturated hydrocarbon groups as well as residual carbonyl and carboxylic acid groups [32]. Both squalane and Apiezon should be purified by chromatography over charcoal and alumina before use, and always if colored [31,33]. Exhaustive hydrogenation can be used to saturate aromatic and olefinic groups to produce a more stable product, Apiezon MH, having properties closer to those of a typical saturated hydrocarbon phase. Apolane-87 is a synthetic hydrocarbon and the most reproducible and thermally stable of the hydrocarbon phases [34]. All hydrocarbon phases are susceptible to oxidation by reaction with the small quantities of oxygen present in

the carrier gas, particularly at elevated temperatures [31,35]. Oxidation alters the chromatographic properties of these phases by introducing polar, oxygenated functional groups and in extreme cases may cause scission of the carbon backbone resulting in a high level of column bleed. Apolane-87 is the most resistant of the common hydrocarbon phases to oxidation because of its low concentration of tertiary hydrogen atoms.

Hydrocarbon phases provide separations based on dispersion interactions with a variable contribution from induction interactions possible when separating polar solutes. For many years they were widely used in the petrochemical industry for speciating volatile hydrocarbon mixtures but have subsequently been replaced by specially synthesized poly(alkylsiloxane) phases containing large alkyl groups [36-38]. Hydrocarbon phases are used as nonpolar reference phases in various schemes proposed to measure the selectivity of polar phases. The method of retention index differences of Rohrschneider and McReynolds (section 2.4.4.2) and the free energy nonpolar interaction term of Kollie and Poole [39] used squalane as a reference phase and the $\log L^{16}$ solute descriptor of the solvation parameter model (section 1.4.3) [40-43] is calculated from correlation equations based on hydrocarbon phases. Hydrocarbon phases have weak-support deactivating properties resulting in unsymmetrical peak shapes and sample-size dependent retention on undeactivated packed column supports [31,42,44]. Even on silanized supports wetting problems persist and a definite transition at a minimum phase loading corresponding to coalescence of the liquid droplets to a continuous film may be observed [31,45]. WCOT columns coated with hydrocarbon phases demonstrate poor temperature stability and limited lifetimes.

Sporadic accounts of the use of fluorocarbon liquid phases for the separation of reactive compounds or speciation of perfluorocarbons and Freons have appeared almost from the inception of gas chromatography [46-48]. Reactive chemicals such as metal fluorides, halogens, interhalogen compounds, and the halide compounds of hydrogen, sulfur, and phosphorus tend to destroy conventional phases. The strong carbon-fluorine bonds in highly fluorinated phases provide the necessary chemical resistance for these applications. Highly-fluorinated phases also possess greater selectivity for the separation of isomeric perfluorocarbons and Freons. The low cohesive energy of perfluorocarbon stationary phases compared with the corresponding hydrocarbon phases results in significant deviations from Raoult's law for fluorocarbon/hydrocarbon mixtures. Consequently, for highly fluorinated stationary phases retention will be less than that for conventional phases, allowing an extension of the molecular weight separating range of gas chromatography or the separation of thermally labile substances at lower temperatures [46,49]. The same weak intermolecular interactions are responsible for the poor support/wall wetting characteristics, low column efficiencies and low maximum operating temperature limits characteristic of early highly fluorinated stationary phases. These deficiencies can be overcome by modifying the structure of the phase to contain anchor groups capable of strong support/wall interactions. Typical of these modified phases are the poly(perfluoroalkyl ether) Fomblin oils [50,51], the fluorinated alkyl esters [52] and poly(diphenyl/1H,1H,2H,2H-perfluorodecylmethyl)siloxane phases [49]. The latter

phase can be immobilized and bonded to the wall of glass capillary columns allowing temperatures up to 400°C to be used, for example, for the separation of beeswax.

2.3.2 Ether and Ester Stationary Phases

The dialkyl phthalates and tetrachlorophthalates are moderately polar and weakly hydrogen-bond basic liquid phases [53]. Phthalate esters with long alkyl chains are the less volatile but have lower selectivity for polar interactions. Phthalate esters with octyl to dodecyl alkyl chains are a reasonable compromise between the desire for a wide temperature operating range and useful solvent selectivity, Table 2.3. Still, their high volatility compared to other phases and limited selectivity for polar interactions have reduced their general importance. Earlier claims that tetrachlorophthalates form strong charge-transfer complexes with electron-donor compounds are probably not well founded.

The term polyester is used to describe a wide range of resinous composites derived from the reaction of a polybasic acid with a polyhydric alcohol [54]. The most frequently used polyester phases for gas chromatography are the succinate and adipate esters of ethylene glycol, diethylene glycol and butanediol, Table 2.3. These phases are used almost entirely in packed column gas chromatography for the separation of polar compounds. Changes in polymer composition by column conditioning, low tolerance to oxygen and water (particularly above 150°C), and solute exchange reactions involving the polyester functional groups and alcohols, acids, amines and esters have contributed to their diminished use in recent years. They have been replaced in many of their former applications by the more stable poly(cyanoalkylsiloxane) phases.

The meta-linked poly(phenyl ethers) are moderately polar liquid phases with a defined chemical structure. Their low volatility is exceptional for low molecular weight liquids. The five and six ring poly(phenyl ethers) have useful operating temperature ranges up to 200°C and 250°C, respectively. Poly(phenyl ethers) containing polar functional groups are less useful liquid phases due to poor column efficiencies and low thermal stability [55]. Copolymerization of the five or six ring poly(phenyl ethers) with diphenyl ether-4,4'-disulfonyl chloride yields a polyether sulfone of undefined molecular weight and chemical structure suitable for the separation of a wide range of high-boiling polar compounds at temperatures in the range 200-400°C [56].

Poly(ethylene glycols) have been widely used for the separation of volatile polar compounds, such as flavor and fragrance compounds, and fatty acid esters [57]. Carbowax 20M is the most popular phase for packed column separations, Table 2.3. It is a waxy solid with a molecular weight of 14,000 to 18,000 melting at about 60°C to a stable liquid with a maximum operating temperature of about 225°C [58]. Specially purified poly(ethylene glycols) of higher molecular weight such as Superox-4 (molecular weight 4 million) [59] and Superox-20M [59,60] can be used at temperatures up to about 250-275°C. Pluronic phases have a lower polydispersity than Carbowax phases and are prepared by condensing propylene oxide with propylene glycol [61]. The resulting chain is then extended on both sides by the addition of controlled amounts

of ethylene oxide until the desired molecular weight is obtained. Pluronic liquid phases with average molecular weights of 2000 to 8000 are most useful for gas chromatography and can be used at temperatures up to about 220-260°C. Condensing Carbowax 20M with 2-nitroterephthalic acid produces a new phase, FFAP, that has been recommended for the separation of organic acids. This phase is unsuitable for the separation of basic compounds or aldehydes with which it reacts. The poly(ethylene glycols) are rapidly degraded by oxygen and moisture at high temperatures. Strong acids and Lewis acid catalysts may also degrade these polymers.

Carbowax 20M was one of the earliest phases used routinely in WCOT columns because of its good coating characteristics. The thermal decomposition products of Carbowax 20M provided a popular method of surface deactivation for subsequent coating with Carbowax 20M and other phases in the early development of capillary column technology [62]. The mechanism of Carbowax deactivation remains unknown, although it is believed that the degradation products bond chemically to the surface silanol groups at the high temperatures employed for deactivation. The method is not widely used today. The improved stability of modern poly(ethylene glycol) WCOT columns towards water and oxygen and the availability of higher operating temperatures is achieved through bonding the polymer to the column wall and/or through crosslinking. For commercial columns the details remain propriety information but some general clues are contained in the literature [61,63-65]. Poly(ethylene glycols) are more difficult to immobilize by free radical crosslinking than the poly(siloxane) phases. Immobilization is possible using "autocrosslinkable" poly(ethylene glycols), free radical crosslinking of copolymers of poly(ethylene glycol) and vinylsiloxanes or by acid catalyzed reaction with an alkyltrialkoxysilane coupling agents. The columns are coated with the poly(ethylene glycol) and necessary reagents and the immobilization achieved by simply heating the column in an inert atmosphere to between 150-200°C for the required time. Crosslinking partially destroys the crystallinity of the poly(ethylene glycol) polymer resulting in a lower column operating temperature and improved film diffusion properties. Diffusion in immobilized poly(ethylene glycol) phases is still less than that observed for immobilized poly(siloxane) phases and thinner films are often selected in practice to maintain acceptable separation efficiency.

2.3.3 Liquid Organic Salts

The liquid organic salts are a novel class of ionic liquids with good solvent properties for organic compounds [66-69]. The alkylammonium and alkylphosphonium salts with weak nucleophilic anions form thermally stable liquids with a low vapor pressure at temperatures exceeding their melting points by 150°C or more in favorable cases, Table 2.3. In addition, a number of these salts have low melting points including several that are liquid at room temperature. For the low molecular weight tetraalkylammonium salts the lowest melting points are often found for the tetrabutylammonium salts. Weak nucleophilic anions such as the sulfonate and tetrafluoroborate anions are generally the most stable and provide the widest liquid operating temperature range.

The liquid organic salts have favorable mass transfer properties and the efficiency of column packings prepared with liquid organic salts are not obviously different to those prepared from conventional non-ionic phases. The liquid organic salts possess low chemical reactivity and transformation reactions are relatively rare [66,70]. Nucleophilic displacement can occur for alkyl halides, degradation of alkanethiols was observed for some salts, and proton transfer and other acid/base reactions can affect the recovery of amines. The unique selectivity of the liquid organic salts is a result of their strong hydrogen-bond basicity and significant capacity for dipole-type interactions. In this respect their solvent properties cannot be duplicated by common non-ionic liquid phases. The retention properties of the salts can be correlated with the structure of the ions, particularly the extent of charge delocalization and intramolecular association, providing the possibility of designing salts for specific applications [68]. Liquid organic salts do not wet glass surfaces well but can be coated on whisker walled and sodium chloride modified glass surfaces for the preparation of WCOT columns. The low viscosity of the liquid organic salts restricts the range of available film thicknesses and the accessible temperature operating range of WCOT columns [66]. No mechanism has been indicated for the preparation of immobilized liquid organic salt columns.

2.3.4 Poly(siloxane) Stationary Phases

The poly(siloxanes) are the most popular liquid phases for gas chromatography. A concert of favorable properties that includes a wide temperature operating range (low temperature glass transition point, low vapor pressure and high thermal stability), acceptable diffusion and solubility properties for different solute types, chemical inertness, good film forming properties, ease of synthesis with a wide range of chromatographic selectivity, and ease of immobilization for applications employing open tubular columns largely accounts for their dominant position in column technology [30,71,72]. The poly(siloxanes) used in gas chromatography are generally linear polymers with structures given in Figure 2.2, in which R can be a number of different substituents such as methyl, vinyl, phenyl, 3,3,3-trifluoropropyl, or 3-cyanopropyl. By varying the identity and relative amount of each R group as well as the ratio of the monomer units n to m, polymers with a wide range of solvent properties can be prepared.

The poly(siloxane) polymers are usually prepared by the acid or base hydrolysis of appropriately substituted dichlorosilanes or dialkoxysilanes, or by the catalytic polymerization of small ring cyclic siloxanes [71-75]. The silanol-terminated polymers are suitable for use after fractionation or are thermally treated to increase molecular weight and in some cases endcapped by trimethylsilyl, alkoxy or acetyl groups [76,77]. Poly(siloxanes) synthesized in this way are limited to polymers that contain substituent groups that are able to survive the relatively harsh hydrolysis conditions, such as alkyl, phenyl, 3,3,3-trifluoropropyl groups. Hydrosilylation provides an alternative route to the synthesis of poly(siloxanes) with labile or complicated substituents (e.g. cyclodextrin, oligoethylene oxide, liquid crystal, amino acid ester, and alcohol) [78-81]. In this case

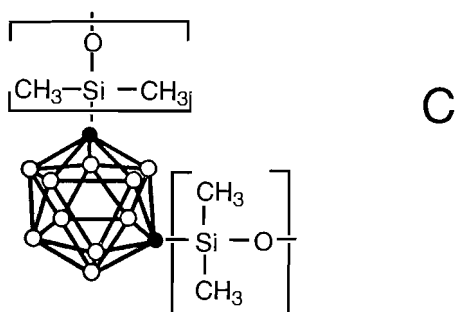
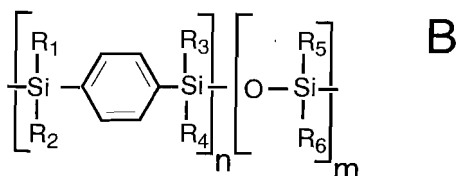
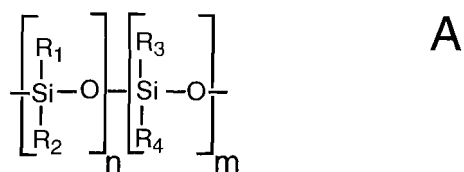


Figure 2.2. General structures of poly(siloxane) liquid phases. A. poly(siloxane) polymer; B. poly(silarylene-siloxane) copolymer; and C, a poly(carborane-siloxane) copolymer (● = carbon and ○ = BH).

synthesis starts with a poly(alkylhydrosiloxane) of the desired molecular weight in which the required fraction of R groups on the poly(siloxane) backbone are hydrogen. The substituent group to be incorporated into the polymer with a sidearm alkene group is then attached to the polymer at each Si-H bond by catalytic (chloroplatinic acid) hydrosilylation under relatively mild conditions. Condensation of carbosilanes and oligodimethylsiloxanolate salts in aprotic solvents provides another route for the preparation of poly(siloxanes) with labile substituents [82]. The reactive carbosilane groups (e.g. isocyanate, epoxy, activated carbonyl esters, etc.) provide an attachment point for the desired substituents by means of secondary reactions through formation of ester, amide, carbamate bonds, etc. Materials of high purity, particularly with regard to the presence of residual catalyst, and of narrow molecular weight range are required for chromatographic purposes. Although many methods are used for characterizing poly(siloxanes), multinuclear NMR is the most informative, providing information about the chemical composition, end group type, average molecular weight, branching, and the backbone microstructure [83].

Table 2.4

Characteristic properties of some poly(siloxane) liquid phases used for packed column gas chromatography

Name	Structure	Viscosity (cP)	Average molecular weight	Temperature operating range (°C)	
				Minimum	Maximum
OV-1	Dimethylsiloxane	gum	> 10 ⁶	100	350
OV-101	Dimethylsiloxane	1500	30,000	<20	350
OV-7	Phenylmethyldimethylsiloxane 80 % methyl and 20 % phenyl	500	10,000	<20	350
OV-17	Phenylmethylsiloxane 50 % methyl and 50 % phenyl	1300	40,000	<20	350
OV-25	Phenylmethyldiphenylsiloxane 25 % methyl and 75 % phenyl	>100,000	10,000	<20	300
OV-210	Trifluoropropylmethylsiloxane 50 % methyl and 50 % 3,3,3-trifluoropropyl	10,000	200,000	<20	275
OV-225	Cyanopropylmethylphenylmethylsiloxane 50 % methyl, 25 % phenyl and 25 % 3-cyanopropyl	9000	8,000	<20	250
Silar 7CP	Cyanopropylphenylsiloxane 75 % 3-cyanopropyl and 25 % phenyl			50	250
OV-275	Di(cyanoalkyl)siloxane 70 % 3-cyanopropyl and 30 % 2-cyanoethyl	20,000	5,000		250
Silar 10CP	Di(3-Cyanopropyl)siloxane			50	250

The gradual evolution of column technology revealed that the low to medium viscosity poly(siloxane) oils preferred as packed column stationary phases, Table 2.4, were unsuitable for preparing thermally stable and efficient open tubular columns. Gum phases of generally higher molecular weight, but more importantly higher viscosity, were required for the preparation of stable films resistant to disruption at elevated temperatures. Poly(dimethylsiloxanes) have a coiled helical structure with their methyl groups pointing outwards. Increasing temperature causes the mean intermolecular distance to increase but at the same time expansion of the helices occurs, tending to diminish this distance. As a result, the viscosity appears to be only slightly affected by temperature. For poly(siloxanes) containing bulky or polar functional groups, the regular helical conformation of the polymers is distorted resulting in a greater change in viscosity with temperature. These phases form films that are not as resistant to temperature variations as the poly(dimethylsiloxanes) but are generally significantly more stable than other types of common liquid phases.

A second important breakthrough in open tubular column technology was the realization that crosslinking of gum phases to form a rubber provided a means of further stabilizing the poly(siloxane) films without destroying their favorable diffusion characteristics. The unmatched flexibility of the silicon oxygen bond imparts great mobility to the polymer chains, providing openings that permit diffusion, even for crosslinked phases. Modern stationary phases used to prepare open tubular columns are characterized by high viscosity, good diffusivity, low glass transition temperatures and are often suitable for crosslinking. For the present suitable properties are only apparent for the poly(siloxane) and poly(ethylene glycol) phases, Table 2.5, explaining how

Table 2.5

Rough guide to the temperature operating range for bonded poly(siloxane) stationary phases in open tubular columns

Type	Temperature Range (°C)		High Temperature Version
	Minimum	Maximum	
Dimethylsiloxane	-60	325	420
Dimethyldiphenylsiloxane (5 % diphenyl)	-60	325	420
Dimethyldiphenylsiloxane (35 % diphenyl)	40	300	340
Dimethyldiphenylsiloxane (50 % diphenyl)	40	325	390
Methylphenylsiloxane	0	280	
Dimethyldiphenylsiloxane (65 % diphenyl)	50	260	370
3,3,3-Trifluoropropylmethylsiloxane (50 % trifluoropropyl)	45	240	300
3-Cyanopropylphenyldimethylsiloxane (6 % cyanopropylphenyl and 84 % dimethyl)	20	280	
3-Cyanopropylphenyldimethylsiloxane (25 % cyanopropylphenyl and 75% dimethyl)	40	240	
3-Cyanopropylphenyldimethylsiloxane (50 % cyanopropylphenyl and 50 % dimethyl)	40	230	
3-cyanopropyl-silphenylene co-polymer (equivalent to 70 % dicyanopropyl)			290
Poly(ethylene glycol)	20	250	280
FFAP	40	250	

they have come to dominate the practice of open tubular column gas chromatography, even more so than the poly(siloxane) phases dominate the field of packed column gas chromatography.

Two different approaches have been used to immobilize poly(siloxane) phases for the preparation of open tubular columns. Thermal immobilization of silanol-terminated non-polar and moderately polar poly(siloxanes) [73,74,84-86] and radical initiated crosslinking of endcapped poly(siloxanes) of a wider polarity range [62,78-80]. Carboxyalkyl-functionalized poly(siloxane) copolymers are also readily immobilized by thermal treatment but are not considered here [87]. For thermal condensation the acid leached and perhaps deactivated glass surface is statically coated with a film of the silanol-terminated polymer (occasionally methoxy-terminated polymers are also used). The column is then heated to 300-370°C for various times (5 to 15 h) in the presence of a slow flow of carrier gas. Thermal induced reactions result in simultaneous bonding of the polymer to the column wall and the formation of crosslinks between polymer chains. Addition of a few percent of a crosslinking agent, such as an alkyltrimethoxysilane, is sometimes used to improve the thermal stability of the film by promoting additional crosslinking and surface bonding.

The crosslinking reaction indicated above is important for another reason. It is also the root cause of thermal degradation of poly(siloxanes) resulting in column bleed at high temperatures. The main degradation products from the heating of silanol-terminated poly(siloxanes) have been identified as hydrocarbons and small ring cyclic

siloxanes [72,84,85]. The formation of hydrocarbons is accounted for by a reaction leading to chain branching identical to that responsible for crosslinking in the column preparation step. The silanol group of one polymer chain acting as a nucleophile to displace the alkyl group from a neighboring polymer chain with the joining of the two chains by a new siloxane bond (crosslink). Similarly, reaction of the terminal silanol groups with surface-bonded organosiloxane groups (formed during deactivation by high temperature silanization) results in covalent bonding of the phases to the column wall. On undeactivated glass surfaces it is probably the reaction of silanol groups on the glass surface with siloxane groups of the polymer backbone that is responsible for surface bonding rather than condensation of polymer and surface silanol groups. Above some critical temperature the generation of crosslinks will be too high and the resulting film will have inadequate solvation and diffusion properties to be useful for gas chromatography. Cyclics are formed by an intramolecular displacement in which the silanol end group attacks a silicon atom in the same chain setting free small cyclic siloxanes with formation of a new silanol group. The cyclic siloxanes released are mainly tri- and tetrameric fragments easily identified by mass spectrometry in the column bleed profile of poly(siloxane) phases. Poly(siloxanes) containing electron-withdrawing cyano groups on α -alkylcarbon or fluorine on a α - or β -alkylcarbon atoms have low thermal stability and are less useful for gas chromatography than substituents with an additional methylene group in the alkyl chain between silicon and the polar functional group. The replacement of a methyl group by phenyl increases the thermal stability of the poly(siloxanes). This results from a strengthening of the siloxane bond by increased participation of the lone pair electrons on oxygen but the silicon aryl bond is more polarized and should be easier to cleave in chemical reactions, such as those leading to crosslinking. Poly(siloxanes) containing 3-cyanopropyl groups are more thermally stable when a phenyl group is attached to the same silicon atom than when a methyl group is present.

The use of silanol-terminated poly(siloxanes) have been instrumental in the development of columns for high temperature gas chromatography, Table 2.5 [88-93]. This requires phases of exceptional thermal stability and starts with meticulous care in the preparation of the glass surfaces to minimize catalytic decomposition of the poly(siloxanes). High molecular weight polymers optimize the number of silanol groups per unit volume of polymer to produce the desired number of crosslinks and minimize the formation of cyclic siloxanes. The thermal stability of the poly(siloxanes) is improved if phenyl groups (silylene copolymers) or carborane groups are incorporated into the poly(siloxane) backbone (Figure 2.2), the substitution on the poly(siloxane) backbone is symmetrical, and the sequence of silyl groups along the siloxane backbone is alternating. For example, a poly(methylphenylsiloxane) containing dimethylsiloxane and diphenylsiloxane units is likely to be more stable than a polymer containing methylphenylsiloxane units in its structure. Incorporating phenyl or carborane groups in the polymer backbone inhibits the formation of cyclic siloxanes by reducing the flexibility of the polymer chains. An example of the use of high temperature gas chromatography to separate hydrocarbons with a carbon number greater than 100 using tempera-

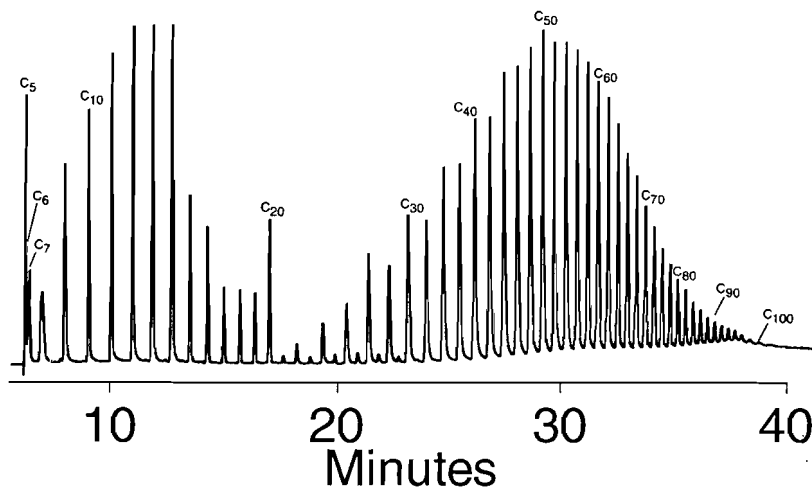


Figure 2.3. Separation of polywax 655 by high temperature gas chromatography on a 6 m x 0.53 mm I. D. open tubular column coated with a 0.1 μm film of a poly(carborane-siloxane) copolymer (equivalent to 5 % phenyl). Initial column temperature -20°C for 1 min, programmed at $10^{\circ}\text{C}/\text{min}$ to 430°C , and final hold 5 min at 430°C . The helium carrier gas flow rate was 20 ml/min. (©SGE, Inc.)

ture programming up to a final temperature of 420°C is presented in Figure 2.3. High temperature gas chromatography is an important technique for the structural analysis of hydrocarbons and triglycerides of high carbon number [88,93-95].

The second general approach to immobilization of poly(siloxanes) is by free radical crosslinking of the polymer chains using peroxides [96-98], azo-compounds [80,97,99] or γ -radiation [100,101] as free radical generators. In this case, crosslinking occurs through the formation of carbon-carbon bonds involving the organic substituents on neighboring poly(siloxane) chains. The silicon atoms are not involved in the crosslinking reaction and no new siloxane bonds are formed. Very little crosslinking (0.1-1.0%) is required to immobilize long chain poly(siloxanes). Dicumyl peroxide and azo-*tert*-butane have emerged as the most widely used free radical generators for stationary phase immobilization.

Free radical crosslinking provides a relatively simple column preparation procedure. The deactivated glass surface is statically coated with a freshly prepared solution of the stationary phase containing 0.2 to 5% w/w of dicumyl peroxide or the coated column is saturated with azo-*tert*-butane vapors entrained in nitrogen gas at room temperature for about 1 h. It is very important that the stationary phase is deposited as an even film since this film will be fixed in position upon crosslinking and no improvement in film homogeneity can be obtained after fixation. The coated column is sealed and slowly raised to the curing temperature for static curing. The curing temperature is a function of the thermal stability of the free radical generator and is selected to give a reasonable half-life for the crosslinking reaction. Once the column

reaches the curing temperature, maintaining that temperature for a short time is all that is required to complete the reaction. Dynamic curing with a slow flow of carrier gas is sometimes used with dicumyl peroxide. After curing, any phase that is not immobilized is rinsed from the column by flushing with appropriate organic solvents. Poly(dimethylsiloxane) phases are relatively easy to immobilize by free radical crosslinking, but with increasing substitution of methyl by bulky or polar functional groups the difficulty of obtaining complete immobilization increases. For this reason moderately polar poly(siloxane) phases are prepared with small amounts of vinyl, tolyl or octyl groups attached to the siloxane backbone or as endcapping groups to increase the success of the crosslinking reaction. Even so, the complete immobilization of poly(cyanopropylsiloxane) phases with a high percentage of cyanopropyl groups has proven difficult. Commercially available immobilized poly(cyanopropylsiloxane) phases are either completely immobilized and contain less than about 50% substitution with cyanopropyl groups or they are "stabilized", indicating incomplete immobilization for phases with a higher incorporation of cyanopropyl groups. Stabilized columns usually have greater thermal stability than physically coated columns but are not resistant to solvent rinsing or as durable as immobilized columns.

Another general advantage of immobilization is that it allows the preparation of thick-film open tubular columns. It is very difficult to prepare conventionally coated columns with stable films thicker than about 0.5 μm . Columns with immobilized phases and film thicknesses of 1.0-8.0 μm are easily prepared. Thick-film columns allow the analysis of volatile substances with reasonable retention factors without the need for subambient temperatures. Immobilization also improves the resistance of the stationary phase to phase stripping by large volume splitless or on-column injection and allows solvent rinsing to be used to free the column from non-volatile sample byproducts or from active breakdown products of the liquid phase. Immobilization procedures also provide a useful increase in the upper column operating temperature limit. Thus, immobilization techniques were important not only in advancing column technology but also contributed to advances in the practice of gas chromatography by facilitating injection, detection and other instrumental developments that would have been difficult without them.

2.3.5 Solvation Properties of Stationary Phases

Solvent strength and selectivity are the properties commonly used to classify liquid stationary phases as selection tools for method development in gas chromatography [29,102-104]. Solvent strength and polarity are often used interchangeably and can cause confusion. Polarity is sometimes considered to be the capacity of a stationary phase for dipole-type interactions alone, while more generally solvent strength is defined as the capacity of a stationary phase for all possible intermolecular interactions. The latter definition is quite sensible but unworkable because there is no substance that is uniquely polar that might be used to probe the polarity of other substances. Indirect measurements of polarity, such as those scales related one way or another to the

reluctance of a polar stationary phase to dissolve a methylene group (or n-alkane) can only determine the capacity of the stationary phase for non polar interactions (dispersion and induction). It can never adequately characterize the capacity of a solvent for specific interactions such as orientation and hydrogen bonding, although these interactions contribute to the solvation process of a methylene group in the form of the greater free energy required for cavity formation. A strong hydrogen-bond acid stationary phase with weak hydrogen-bond basicity, for example, will undergo weaker interactions with itself (solvent-solvent interactions) than with a solute that is a strong hydrogen-bond base. In this case the solvation process for a methylene group will not adequately characterize the capacity of the stationary phase for hydrogen bond formation. Because of these considerations and others it is necessary to abandon the use of stationary phase classification based on a single parameter, indicated as polarity, in spite of the obvious emotive attributes of such a simple scale.

The selectivity of a stationary phase is defined as its relative capacity for specific intermolecular interactions, such as dispersion, induction, orientation and complexation (including hydrogen bond formation). Unlike solvent strength (polarity) it should be feasible to devise experimental scales of stationary phase selectivity. Early attempts to define selectivity scales were based on the system of phase constants introduced by Rohrschneider and subsequently modified by McReynolds (section 2.4.4.2), Snyder's solvent selectivity triangle [105], Hawkes polarity indices [106], solubility parameters [107], and the Gibbs free energy of solution for selected solutes or functional groups [39,108]. These approaches are reviewed in detail elsewhere [28-30,102,103,109,110], but are no longer relevant for stationary phase classification.

Modern approaches to stationary phase classification by selectivity ranking are based on the cavity model of solvation [102,104,111,112]. This model assumes that the transfer of a solute from the gas phase to solution in the stationary phase involves three steps. Initially a cavity is formed in the stationary phase identical in volume to the solute. The solute is then transferred to the cavity with reorganization of the solvent molecules around the cavity and the set up of solute-solvent interactions. The individual free energy terms involved in the transfer are assumed to be additive. In addition, it can be assumed that the free energy associated with cavity reorganization is small compared to the other processes and that the gas-solute vapors behave ideally. Consequently, gas-solute and solute-solute interactions in the gas phase are negligible. For separation conditions employed in analytical gas chromatography infinite dilution conditions can be assumed so that all solution interactions are of the solvent-solvent and solute-solvent type. These interactions can be characterized as dispersion, induction, orientation and complexation. Dispersion interactions are non-selective and are the binding forces that hold all molecular assemblies together. Molecules with a permanent dipole moment can interact with each other by the co-operative alignment of their dipoles (orientation interactions) and by their capacity to induce a temporary complementary dipole in a polarizable molecule (induction interactions). Complexation interactions are selective interactions involving the sharing of electron density or a hydrogen atom between molecules (e.g. hydrogen bonding and charge transfer). In gas-liquid chromatography

retention will depend on the cohesive energy of the stationary phase represented by the free energy required for cavity formation in the stationary phase, the formation of additional dispersion interactions of a solute-solvent type, and on selective solute-solvent polar interactions dependent on the complementary character of the polar properties of the solute and solvent.

The master retention equation of the solvation parameter model relating the above processes to experimentally quantifiable contributions from all possible intermolecular interactions was presented in section 1.4.3. The system constants in the model (see Eq. 1.7 or 1.7a) convey all information of the ability of the stationary phase to participate in solute-solvent intermolecular interactions. The r constant refers to the ability of the stationary phase to interact with solute n - or π -electron pairs. The s constant establishes the ability of the stationary phase to take part in dipole-type interactions. The a constant is a measure of stationary phase hydrogen-bond basicity and the b constant stationary phase hydrogen-bond acidity. The l constant incorporates contributions from stationary phase cavity formation and solute-solvent dispersion interactions. The system constants for some common packed column stationary phases are summarized in Table 2.6 [68,81,103,104,113]. Further values for non-ionic stationary phases [114,115], liquid organic salts [68,116], cyclodextrins [117], and lanthanide chelates dissolved in a poly(dimethylsiloxane) [118] are summarized elsewhere.

The system constants in Table 2.6 are only loosely scaled to each other so that changes in magnitude in any column can be read directly but changes in magnitude along rows must be interpreted cautiously. Most stationary phases possess some capacity for electron lone pair interactions (r constant), but selectivity for this interaction is rather limited among common stationary phases. Fluorine-containing stationary phases have negative values of the r constant representing the tighter binding of electron pairs in fluorocarbon compared to hydrocarbon groups. Electron lone pair interactions do not usually make a significant contribution to retention in gas-liquid chromatography and are not considered as a primary means of selectivity optimization. The most striking feature of Table 2.6 is the paucity of stationary phases with significant hydrogen-bond acidity (b constant). In the case of EGAD, DEGS and TCEP the small b constant is more likely a reflection of impurities in the stationary phase produced during synthesis or while in use [113]. Many stationary phases contain hydrogen-bond acid groups such as hydroxyl, amide or phenol groups that are expected to behave as hydrogen-bond acids. These groups are also significant hydrogen-bond bases and prefer to self-associate forming inter- and intramolecular hydrogen-bond complexes to the exclusion of hydrogen-bond acid interactions with basic solutes. It is only recently that suitable stationary phases with hydrogen-bond acid properties have been described, Figure 2.4 [81,119,120]. Outstanding among these is PSF6 which has a wide temperature operating range and is a strong hydrogen-bond acid with zero hydrogen-bond basicity. These properties reflect the chemistry of the fluorinated alcohol portion of the poly(siloxane) polymer. The strong electron withdrawing trifluoromethyl groups lower the electron density on the neighboring oxygen atom of the hydroxyl group increasing its hydrogen-bond acidity while simultaneously reducing its hydrogen-bond

Table 2.6

System constants derived from the solvation parameter model for packed column stationary phases at 121°C

Stationary phase	System constant					
	<i>r</i>	<i>s</i>	<i>a</i>	<i>b</i>	<i>l</i>	<i>c</i>
<i>(i) Hydrocarbon phases</i>						
Squalane	0.129	0.011	0	0	0.583	-0.222
Apolane-87	0.170	0	0	0	0.549	-0.221
<i>(ii) Ether and ester phases</i>						
Poly(phenyl ether) 5 rings PPE-5	0.230	0.829	0.337	0	0.527	-0.395
Carbowax 20M CW20M	0.317	1.256	1.883	0	0.447	-0.560
Poly(ethylene glycol) Ucon 50 HB 660	0.372	0.632	1.277	0	0.499	-0.184
1,2,3-Tris(2-cyanoethoxypropane) TCEP	0.116	2.088	2.095	0.261	0.370	-0.744
Didecylphthalate DDP	0	0.748	0.765	0	0.560	-0.328
Poly(ethylene glycol adipate) EGAD	0.132	1.394	1.820	0.206	0.429	-0.688
Poly(diethylene glycol succinate) DEGS	0.230	1.572	2.105	0.171	0.407	-0.650
<i>(iii) Liquid organic salts</i>						
Tetrabutylammonium 4-toluenesulfonate QBApTS	0.156	1.582	3.295	0	0.459	-0.686
Tetrabutylammonium tris(hydroxymethyl)methylamino-2-hydroxy-1-propanesulfonate QBATAPSO	0.266	1.959	3.058	0	0.317	-0.860
Tetrabutylammonium 4-morpholinepropane-sulfonate QBAMPS	0	1.748	3.538	0	0.550	-0.937
Tetrabutylammonium methanesulfonate QBAMES	0.334	1.454	3.762	0	0.435	-0.612
<i>(iv) Poly(siloxane) phases</i>						
Poly(dimethylsiloxane) SE-30	0.024	0.190	0.125	0	0.498	-0.194
Poly(dimethylmethylphenylsiloxane) OV-3 (10 mol % phenyl)	0.033	0.328	0.152	0	0.503	-0.181
Poly(dimethylmethylphenylsiloxane) OV-7 (20 mol % phenyl)	0.056	0.433	0.165	0	0.510	-0.231
Poly(dimethylmethylphenylsiloxane) OV-11 (35 mol % phenyl)	0.097	0.544	0.174	0	0.516	-0.303
Poly(methylphenylsiloxane) OV-17	0.071	0.653	0.263	0	0.518	-0.372
Poly(methylphenyldiphenylsiloxane) OV-22 (65 mol % phenyl)	0.201	0.664	0.190	0	0.482	-0.328
Poly(methylphenyldiphenylsiloxane) OV-25 (75 mol % phenyl)	0.277	0.644	0.182	0	0.472	-0.273
Poly(cyanopropylmethyldimethylsiloxane) (10 mol % cyanopropylmethylsiloxane) OV-105	0	0.364	0.407	0	0.496	-0.203
Poly(cyanopropylmethylphenylmethylsiloxane) (50 mol % cyanopropylmethylsiloxane) OV-225	0	1.226	1.065	0	0.466	-0.541
Poly(dicyanoalkylsiloxane) OV-275 (70 mol % dicyanopropyl and 30 mol % dicyanoethyl)	0.206	2.080	1.986	0	0.294	-0.909
Poly(trifluoropropylmethylsiloxane) QF-1	-0.449	1.157	0.187	0	0.419	-0.269
Poly(dimethylsiloxane)-Poly(ethylene glycol) Copolymer OV-330	0.104	1.056	1.419	0	0.481	-0.430
PSF6 (see Fig. 2.4)	-0.360	0.820	0	1.110	0.540	-0.510
<i>(v) Miscellaneous</i>						
Bis(3-allyl-4-hydroxyphenyl)sulfone H10	-0.051	1.323	1.266	1.457	0.418	-0.568

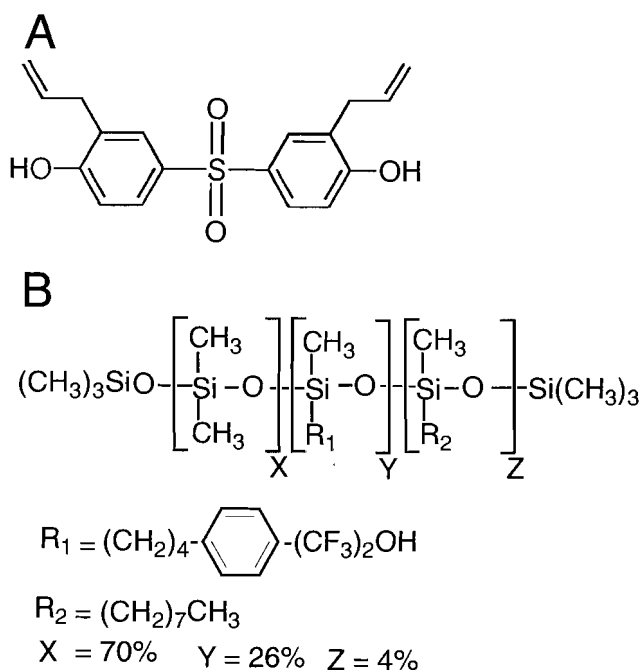


Figure 2.4. Structures of two hydrogen-bond acid stationary phases. A, bis(3-allyl-4-hydroxyphenyl)sulfone (H10) and B, poly(oxy[methyl]4-(2-hydroxy-1,1,1,3,3,3-hexafluoropropyl-2-yl)phenyl]butyl)silylene)-co-oxy(dimethylsilylene) containing 4 mol % octylmethylsiloxane and 70 % dimethylsiloxane (PSF6).

basicity. Because hydrogen-bond acid stationary phases are new to gas chromatography and none are commercially available, there is little experience in their use for selectivity optimization. That leaves the most important stationary phase properties for selectivity optimization as their cohesive energy and capacity for dipole-type and hydrogen-bond base interactions.

The solvation parameter model cannot separate the cohesive solvent-solvent and solute-solvent dispersion interactions since both are strongly correlated to the solute volume. In the general form of the solvation parameter model they are contained in the contribution of the model constant (c term) and in the l term. The model constant term contains other factors besides a contribution from cavity formation, including the phase ratio when the dependent variable is other than the gas-liquid distribution constant (e.g. the retention factor), scaling errors in the solute descriptors, and contributions of a statistical nature from a lack of fit to the model. Proceeding cautiously we can use the product term $\Sigma(c + l \log L^{16})$ to assess the importance of the cavity/dispersion term to retention. Using decane as an example, some data for $\Sigma(c + l \log L^{16})$ are summarized in Table 2.7. In all cases the product term $\Sigma(c + l \log L^{16})$ is positive indicating that the solute-solvent dispersion interactions setup by placing decane into

Table 2.7

Contribution of cavity formation and dispersion interactions to solution of decane in different stationary phases at 121°C

(QBATS = tetrabutylammonium 4-toluenesulfate).

Stationary phase	c	$l \log L^{16}$	$\Sigma(c + l \log L^{16})$
Squalane	-0.222	2.732	2.510
SE-30	-0.194	2.334	2.140
PPE-5	-0.395	2.470	2.075
OV-17	-0.372	2.427	2.055
QF-1	-0.269	1.963	1.694
OV-225	-0.541	2.184	1.643
Carbowax 20M	-0.560	2.095	1.535
QBATS	-0.686	2.151	1.465
DEGS	-0.650	1.907	1.257
TCEP	-0.744	1.734	0.990
OV-275	-0.909	1.378	0.469

the cavity exceed the free energy required to disrupt the solvent structure in forming the cavity. The cavity/dispersion term for the hydrocarbons, poly(dimethylsiloxanes) and poly(methylphenylsiloxanes) are in fact all very similar indicating roughly equal difficulty in forming a cavity in the stationary phase [121]. Stationary phases with dipolar and hydrogen bonding functional groups are considerably more cohesive and the additional free energy required for cavity formation is reflected in the significantly smaller value for $\Sigma(c + l \log L^{16})$. This is particularly so for the poly(cyanoalkylsiloxane) stationary phase OV-275, for which the product term $\Sigma(c + l \log L^{16})$ is considerably smaller than for all other phases in Table 2.7, and the high cohesive energy of this phase is a significant factor in explaining its selectivity. From an interpretive point of view the l constant indicates the spacing between members of a homologous series and contains useful information for phase selection. There is generally a good correlation between the l system constant and the partial molar Gibbs free energy of solution for a methylene group. The liquid organic salts with non-associating anions have surprisingly large l constants compared to non-ionic polar stationary phases, 0.44 to 0.55, and are unique among polar stationary phases in their ability to separate compounds belonging to a homologous series [68]. For anions believed to be associated as hydrogen-bond complexes the l constants are significantly smaller, 0.26 to 0.37, and equivalent to the values observed for the most polar non-ionic stationary phases. An example of the unique selectivity and modest cohesive energy of a non-associated liquid organic salt, tetrabutylammonium 4-toluenesulfonate to separate a test mixture of polar compounds compared to a polar non-ionic phase, OV-275, is shown in Figure 2.5.

The stationary phases in Table 2.6 differ significantly in their capacity for dipole-type interactions (s constant) and in their hydrogen-bond basicity (a constant). For the poly(methylphenylsiloxanes) increasing the phenyl content up to 50 mol % phenyl groups produces an orderly change in the capacity of the phases for dipole-type interactions and a shallow change in their capacity for interactions as a hydrogen-

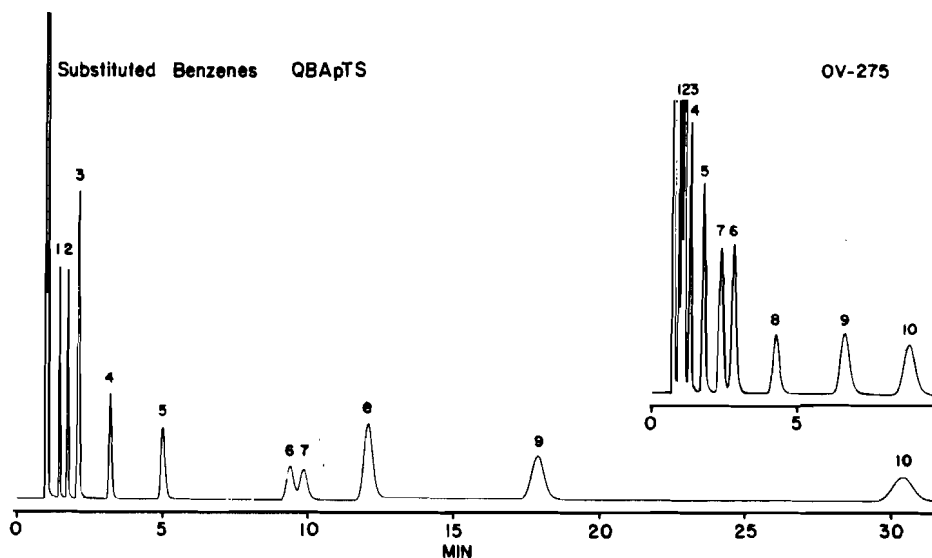


Figure 2.5. Separation of a mixture of polar compounds on matched packed columns coated with tetrabutylammonium 4-toluenesulfonate (QBApTS) and OV-275. Each column was 3.5 m x 2 mm I.D. containing 10% (w/w) of stationary phase on Chromosorb W-AW (100-120 mesh) with a carrier gas flow rate of 15 ml/min and column temperature 140°C. Peak assignments: 1 = benzene; 2 = toluene; 3 = ethylbenzene; 4 = chlorobenzene; 5 = bromobenzene; 6 = iodobenzene; 7 = 1,2-dichlorobenzene; 8 = benzaldehyde; 9 = acetophenone; and 10 = nitrobenzene.

bond base, Figure 2.6. Above 50 mol % phenyl groups there is a slight decline in the system constants as additional phenyl groups are added as diphenylsiloxane monomer units. The r system constant, which is numerically small up to 50 mol % phenyl groups, shows an abrupt increase in value as diphenylsiloxane groups are introduced into the poly(siloxane). Given that the cavity/dispersion term of the poly(methylphenylsiloxanes) changes little with composition, the principal selectivity difference among these phases is due to changes in their capacity for dipole-type interactions. Above 50 mol % phenyl groups selectivity differences are small and result primarily from changes in cohesive energy and lone-pair electron interactions.

Principal component analysis and hierarchical clustering methods provide useful approaches for stationary phase classification [68,81,103,1113,114,121]. The results from principal component analysis for 52 varied non hydrogen-bond acidic stationary phases with their system constants entered as variables are shown in Figure 2.7. The first two principal components account for about 95% of the total variance with the first principal component (PC 1) strongly associated with hydrogen-bond base interactions (a constant) and the second principal component (PC 2) strongly associated with dipole-type interactions and phase cohesive energy (s and l system constants). The stationary phases are classified into three disperse groups. The group 1 stationary phases (e.g.

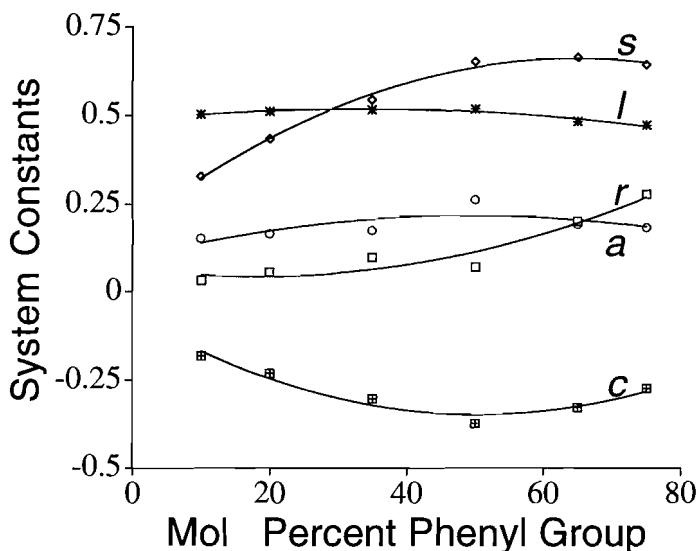


Figure 2.6. Plot of the system constants against the mol % phenyl composition for a series of poly(methylphenylsiloxane) and poly(methylphenyldiphenylsiloxane) phases.

squalane, SE-30, OV-3, OV-7, OV-105, OV-11, OV-17, OV-22, OV-25, PPE-5, QF-1) are weak hydrogen-bond bases with a weak and variable capacity for dipole-type interactions. Differences in cohesive energy are small. Group 2 contains the polar and cohesive non-ionic stationary phases and some liquid organic salts with highly delocalized anions (e.g. U50HB, OV-225, OV-275, OV-330, TCEP, CW 20M, EGAD, DEGS, tetrabutylammonium picrate). These phases have a narrow range of hydrogen-bond basicity and are differentiated primarily by differences in their capacity for dipole-type interactions and their cohesive energy. Group 3 contains only liquid organic salts, which are all dipolar ($s = 1.4$ to 2.1) and strong hydrogen-bond bases ($a = 1.4$ to 5.4) with variable cohesive energy. The non-ionic stationary phases have s and a system constants that each cover the same range of 0 to 2.1. The hydrogen-bond basicity of the liquid organic salts depends primarily on the extent of charge delocalization and anion size. Charge delocalizing anions (e.g. picrate, perfluorobenzenesulfonate) are weaker hydrogen-bond bases and less dipolar than the other salts. Small non-delocalizing anions (e.g. chloride, bromide) are the most hydrogen-bond basic. Anions containing hydrogen bonding substituents have only a small influence on the dipolarity and hydrogen-bond basicity of the salts (s and a constants only slightly changed) but result in a significant increase in their cohesive energy (l constant reduced).

Cluster analysis is an alternative to principal component analysis for the classification of samples by multivariate analysis. The outputs for clustering algorithms are dendrograms. The complete link dendrogram for the stationary phases listed in Table 2.6 is shown in Figure 2.8. The stationary phases most similar to each other are next to each

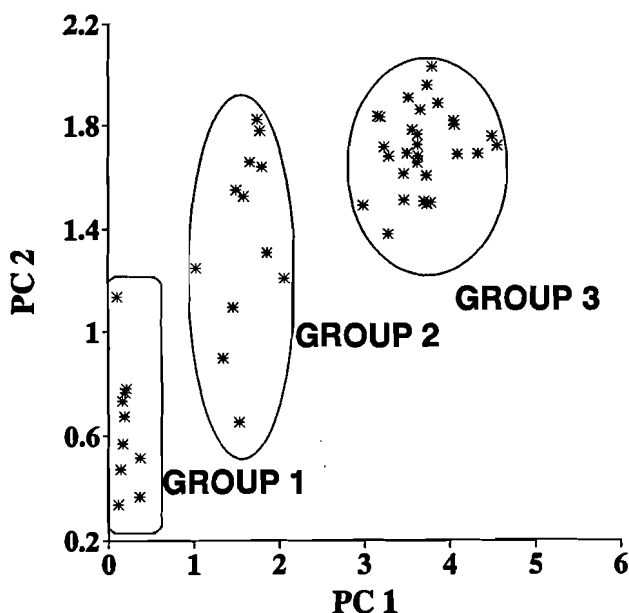


Figure 2.7. Principal component score plot with the system constants from the solvation parameter model as variables for 52 non hydrogen-bond acid stationary phases at 121°C. Loading for PC 1: $0.996 a + 0.059 s - 0.054 l - 0.024 c - 0.014 r$. Loading for PC 2: $0.940 s + 0.328 l + 0.080 r + 0.027 c - 0.037 a$.

other and are connected. Connections at the extreme left-hand side of the dendrogram occur for phases with similar properties and those towards the right-hand side with greater degrees of difference. Stationary phases with no paired descendents are singular phases with properties that cannot be duplicated by other phases in the data set. The stationary phases in Table 2.6 are classified into six groups with three phases behaving independently. Group 1 contains squalane, Apolane-87, OV-3, OV-7, SE-30 and OV-105. These are phases of low cohesive energy with minimal capacity for polar interactions. The second group of stationary phases contains OV-22, OV-25, OV-11, OV-17, PPE-5 and DDP. These phases have low cohesive energy and are weakly dipolar and hydrogen-bond basic. QF-1 is loosely connected to this group but is significantly more dipolar and has a more significant and opposite capacity for lone-pair electron interactions. The third group contains OV-330 and OV-225 with UH50B loosely connected to this group. Compared to the second group these stationary phases are more dipolar and hydrogen-bond basic and slightly more cohesive. They represent an increase in the intensity of the same range of interactions as the larger group of stationary phases. The fourth group contains H10 and PSF6. These are strong hydrogen-bond acid solvents but in other respects quite different to each other. The fifth group contains the liquid organic salts with QBATAPSO distinguished within this group by its greater cohesive energy. Phases in this group are dipolar and strong hydrogen-bond bases as discussed

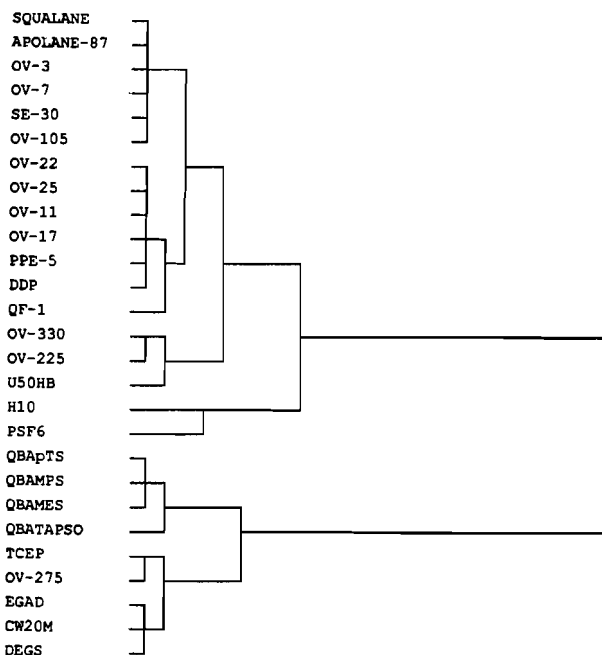


Figure 2.8. Nearest neighbor complete link cluster dendrogram for the stationary phases in Table 2.6. The system constants from the solvation parameter model were used as variables.

earlier. The sixth group of solvents is divided into two subgroups. TCEP and OV-275 are strongly dipolar, hydrogen-bond basic and have high cohesive energy. EGAD, CW20M and DEGS have a similar range of polar interactions, but not quite as intense, and have a lower cohesive energy than TCEP and OV-275. For selectivity optimization in packed column gas chromatography a single phase is initially selected from each group. Subsequently for fine tuning additional phases are selected from within the group identified as possessing the desired separation properties. Stationary phase selection must take into account the temperature operating range for the phases as well as their selectivity.

So far we have discussed solvation properties at a reference temperature of 120°C. The choice of reference temperature arises from historical considerations. McReynolds chose this temperature to compile his extensive database of retention measurements for volatile solutes on a large number of stationary phases. His database has been widely used for exploring new approaches to stationary phase classification and has influenced others into using the same temperature to collect additional reference data to maintain compatibility with the original database. The choice of a standard reference temperature is of less concern than whether a single reference temperature is sufficient to classify solvent properties for use at temperatures distant from the reference temperature. There is only a limited amount of data for the influence of temperature on selectivity in gas-liquid chromatography [53,81,103,121,122]. In general polar interactions are

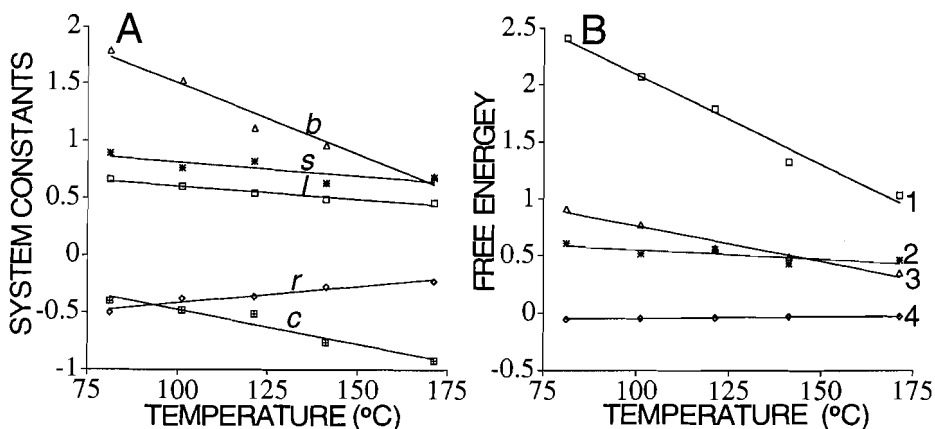


Figure 2.9. Influence of temperature on the system constants (A) and contributions of individual intermolecular interactions to the retention of octan-2-one (B) for the hydrogen-bond acid stationary phase PSF6. For (B) 1 = contribution from cavity formation and dispersion interactions; (2) contribution from dipole-type interactions; (3) contribution from solute hydrogen-bond base and stationary phase hydrogen-bond acid interactions; and (4) contribution from lone pair electron repulsion. Note for PSF6 there are no contributions from stationary phase hydrogen-bond base interactions since the *a* system constant is zero. (From ref. [81]; ©Elsevier)

expected to decrease with increasing temperature, and cavity formation should be easier at higher temperatures, but for individual stationary phases the magnitude of these temperature-induced changes will not be the same. In the solvation parameter model each product term representing a defined interaction can be regarded as made up of a solute factor, a stationary phase factor and a coefficient. In theory all three contributions could be temperature dependent. Out of practical necessity the solute descriptors are considered temperature invariant (otherwise a new set of descriptors would be needed for each temperature used). It is found experimentally that system constants alter quite regularly with temperature, and plots of system constants against temperature (or reciprocal temperature) are linear or shallow curves. The *s*, *a*, *b*, and *l* system constants are found to decline with increasing temperature while *r* is less predictable and often increases with increasing temperature. The use of a hydrocarbon reference phase instead of the gas phase for the R_2 solute descriptor is the likely reason for this difference. Figure 2.9 provides examples of the influence of temperature on the system constants and the contribution of intermolecular interactions (product terms) to the retention of octan-2-one for the hydrogen-bond acid stationary phase PSF6 [81]. There is a significant decline in the contribution of hydrogen-bond acid and lone-pair electron repulsion interactions for the stationary phase but only a modest decline in dipole-type interactions with increasing temperature. By extrapolation PSF6 would be expected to show zero hydrogen-bond acidity at about 210°C ($b = 0$) while remaining significantly dipolar ($s > 0$). Solute hydrogen-bond base stationary phase hydrogen-bond acid interactions exhibit a greater temperature dependence than

dipole-type interactions for octan-2-one such that hydrogen-bond interactions are more important as polar contributions to retention at lower temperatures and dipole-type interactions at higher temperatures. Throughout the temperature range investigated the cavity/dispersion contribution to retention is the most important for octan-2-one with lone-pair electron repulsion interactions of minor significance. Consequently, the capacity of a stationary phase for specific intermolecular interactions determined at a single reference temperature can be quite misleading for selectivity optimization at other temperatures. When stationary phases are ranked in order of their capacity for individual intermolecular interactions at different temperatures crossovers occur. Also, selectivity differences between individual stationary phases are enhanced at low temperatures with phases becoming more alike at higher temperatures. Information on the contribution of polar interactions to retention at high temperatures is scarce. These contributions could be small and stationary phase selectivity differences rather limited at high temperatures.

A large database of system constants for open tubular column stationary phases determined at 20°C intervals over the temperature range 60-140°C is available [43,104,123-127]. The system constants at 120°C are summarized in Table 2.8 for comparison with the packed column stationary phases (Table 2.6). The open tubular column stationary phases show a good coverage of the upper range of the *l* system constant, limited coverage of the *r* system constant range, and near complete coverage of the *s* system constant range of the packed column stationary phases [125]. There is near complete coverage of the *a* system constant range if only the non-ionic packed column stationary phases are considered. Except for the liquid organic salts, there is reasonable coverage of the selectivity range of the more chemically diverse packed column stationary phases by the open tubular column stationary phases.

Multiple entries in Table 2.8 for generic column types allow an assessment of the selectivity equivalence of nominally identical columns from different manufacturers. Five poly(dimethyldiphenylsiloxane) columns containing 5% diphenylsiloxane monomer (PMPS-5 in Table 2.8) exhibited virtually equivalent selectivity with minor differences in hydrogen-bond basicity [124]. Selectivity differences between the arylene-siloxane copolymer (AS-5 in Table 2.8) and the PMPS-5 stationary phases are somewhat larger, with AS-5 being less cohesive and more hydrogen-bond basic than the PMPS-5 stationary phases. Small, although significant selectivity differences, were noted among the poly(ethylene glycol) stationary phases (PEG in Table 2.8) [123]. These selectivity differences were indicated to result from chemical differences among the stationary phases and from differences in the relative contribution of interfacial adsorption to the retention mechanism (section 2.4.1). The latter depends on both system properties (film thickness and column radius) and solute characteristics.

Most of the stationary phase types in Table 2.8 were derived from popular packed column stationary phases and subsequently modified to allow for immobilization and improved thermal stability. Their selectivity equivalence can be ascertained by comparison of system constants for stationary phases of nominally similar chemical composition in Table 2.6 and 2.8 [125]. There are small differences in selectivity for

Table 2.8

System constants for open tubular column stationary phases at 120°C

(Dependent variable log k)

General abbreviation	Column identity	System constants ($b = 0$ in all cases)			
		l	r	s	a
<i>Poly(dimethylsiloxane)</i>					
PMS	DB-1	0.504	0	0.207	0.185
<i>Poly(methyloctylsiloxane)</i>					
PMOS	SPB-Octyl	0.615	0	0.232	0
<i>Poly(dimethyldiphenylsiloxane)</i>					
PMPS-5	DB-5	0.513	0	0.280	0.193
	HP-5	0.518	0	0.309	0.205
	OV-5	0.503	0	0.286	0.223
	SPB-5	0.504	0	0.293	0.212
	PTE-5	0.505	0	0.293	0.210
PMPS-20	Rtx-20	0.549	0	0.564	0.259
PMPS-35	DB-35	0.540	0	0.695	0.314
PMPS-50	HP-50+	0.474	0.160	0.623	0.281
	Rtx-50	0.519	0.057	0.796	0.339
PMPS-65	Rtx-65	0.531	0.108	0.839	0.358
<i>Arylene-Siloxane copolymer (nominally similar to HP-5)</i>					
AS-5	HP-5TA	0.595	0	0.350	0.284
<i>Poly(methyltrifluoropropylsiloxane)</i>					
PMTS-20	DB-200	0.464	-0.340	1.010	0.203
PMTS-50	DB-210	0.439	-0.343	1.278	0.077
<i>Poly(cyanopropylmethylsiloxane)</i>					
PCM-50	DB-23	0.438	0	1.537	1.468
<i>Poly(cyanopropylphenyldimethylsiloxane)</i>					
PCPM-06	DB-1301	0.547	-0.057	0.498	0.450
PCPM-14	DB-1701	0.494	-0.066	0.667	0.643
PCPM-50	DB-225	0.444	-0.051	1.249	1.110
<i>Poly(cyanopropylsiloxane)</i>					
PCPS	SP-2340	0.418	0	1.993	1.960
<i>Poly(ethylene glycol)</i>					
PEG	HP-INNOWax	0.458	0.219	1.351	1.882
	HP-20M	0.452	0.209	1.335	2.014
	AT-Wax	0.440	0.225	1.318	1.889
NPEG	DB-FFAP	0.428	0.214	1.424	2.077
<i>Poly(siloxane) of unknown composition</i>					
VRX	DB-VRX	0.543	0	0.304	0.159
<i>Mixtures of poly(dimethylsiloxane) and poly(ethylene glycol)*</i>					
DX-1	DX-1	0.536	0	0.384	0.465
DX-3	DX-3	0.539	0.148	1.000	1.632
DX-4	DX-4	0.508	0.209	1.247	1.786

* DX-1 = 10%, DX-3 = 50% and DX-4 = 85% poly(ethylene glycol)

the polar phases and the description “of similar” rather than “equivalent” selectivity is justified. Some of these differences have a chemical origin, but a part of these differences might be explained by contributions from interfacial adsorption. The data in Table 2.6 are corrected for interfacial adsorption while the results in Table 2.8 are not.

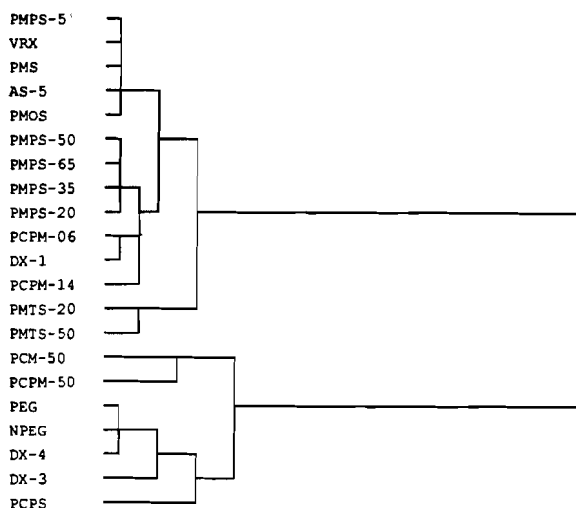


Figure 2.10. Average linkage (within group) cluster dendrogram for the stationary phases in Table 2.8. An average value is used for chemically similar stationary phases. (From ref. [127]; ©John Wiley & Sons).

The poly(siloxane) stationary phases in Table 2.8 span a wide range of selectivity, which is generally engineered by replacing dimethylsiloxane monomer groups by monomer groups containing diphenylsiloxane (PMPS), 3,3,3-trifluoropropylmethylsiloxane (PMTS) and various monomers containing a cyanoalkyl substituents (PCM, PCPM and PCPS). The incorporation of diphenylsiloxane monomer groups into the poly(dimethylsiloxane) backbone primarily increases the dipolarity/polarizability (s system constant) for the phase with a small change in hydrogen-bond basicity and cohesion. The change in system constants with composition is nearly linear for phases containing less than 50% diphenylsiloxane monomer groups and is non-linear and smaller at higher levels of diphenylsiloxane incorporation. Plots of the system constant against temperature are approximately linear with the system constants smaller at higher temperatures. The incorporation of 3,3,3-trifluoropropylmethylsiloxane monomer groups produces a significant change in the dipolarity/polarizability of the phase with little change in the hydrogen-bond basicity. This results in a unique change in the s/a system constant ratio and phase selectivity. The introduction of monomers containing cyanoalkyl groups increases the capacity of the phase for dipole-type interactions as well as its hydrogen-bond basicity. This results in a different value for the s/a system constant ratio and a different selectivity to phases containing 3,3,3-trifluoropropylmethylsiloxane monomer groups [125]. In general, incorporation of monomer groups containing polar functional groups results in an increase in the cohesion of the stationary phase, reflected in a smaller value for the l system constant.

Hierarchical cluster analysis, Figure 2.10, of the stationary phases in Table 2.8 provides a convenient tool to visualize selectivity relationships. Two large clusters

containing all the low polarity stationary phases (PMPS-5, VRX, PMS, AS-5 and PMOS) and the poly(dimethyldiphenylsiloxane) phases containing more than 5% diphenylsiloxane monomer groups (PMPS-50, PMPS-65, PMPS-35 and PMPS-20) are observed. Phases in the second cluster have a greater capacity for dipole-type interactions and are more hydrogen-bond basic than those in the first cluster. Their nearest neighbors are the moderately polar poly(cyanopropylphenyldimethylsiloxane) phases with a low incorporation of cyanopropylphenylsiloxane monomer groups (PCPM-06 and PCPM-14) and the mixed poly(dimethylsiloxane) and poly(ethylene glycol) stationary phase DX-1. For a large and varied group of compounds, it was shown that PCPM-6 could replace the stationary phase mixture, DX-1, for all but the most critical separations, supporting the conclusions from cluster analysis [127]. The moderately polar PMTS-20 and PMTS-50 phases are more dipolar/polarizable and cohesive than the phases discussed above with a different selectivity for electron lone pair interactions. PCM-50 and PCPM-50 are cohesive, dipolar and hydrogen-bond basic stationary phases with more in common with the poly(ethylene glycol) phases than the other poly(siloxane) phases in the database. PCPS is the most cohesive, dipolar and hydrogen-bond basic of the stationary phases and is indicated as a singular phase in Figure 2.10. For method development PMS (or PMOS, PMPS-5), PMPS-50, PEG, DX-3 and PCPS are suitable phases for selectivity screening. Once a suitable general selectivity is identified, phases within that group are explored for selectivity optimization. Phases within a group are of a similar but not equivalent selectivity. Consequently, exploring different phases within a group provides a powerful approach for optimizing band spacing.

2.3.6 Solid Stationary Phases

Solid stationary phases are used in gas-solid chromatography where the presumed retention mechanism is dominated by surface adsorption interactions. Gas-solid chromatography is used for a narrow range of applications, which can be characterized as those difficult to achieve by gas-liquid chromatography above ambient temperature. Typical examples include the separation of fixed gases, volatile hydrocarbons, halocarbons, solvents and sulfur gases (compounds containing ≤ 12 carbon atoms and with a boiling point $< 200^\circ\text{C}$). In addition, adsorbents with immobilized active centers are useful for the separation of isomers and isotopomers. For these applications the presence of surface features with a rigid spatial arrangement are important and represent the distinguishing properties of adsorbents compared with liquids.

Some of the less attractive features of gas-solid chromatography include non-linear adsorption isotherms, even for small sample sizes, causing sample size dependent retention and asymmetric peak shapes. Adsorbents with chemically active functional groups can irreversibly adsorb or bond some sample types resulting in their partial or complete abstraction from the chromatogram. The high adsorption energy and large surface areas presented by most common adsorbents results in excessive retention for high boiling and polar compounds. Mass transfer kinetics, and consequently column

Table 2.9

General applications of PLOT columns in gas chromatography

Q = Poly(divinylbenzene-styrene), S = poly(divinylbenzene-vinylpyridine) and U = poly(divinylbenzene-ethylene glycol dimethacrylate)

Stationary phase	Maximum operating temperature (°C)	Typical applications
Alumina oxide	200	Alkanes, alkenes, alkynes and aromatic hydrocarbons from C ₁ to C ₁₀ . C ₁ and C ₂ halocarbons
Silica gel	250	Hydrocarbons (C ₁ to C ₄), inorganic gases, volatile ethers, esters and ketones
Carbon	350	Inorganic gases, hydrocarbons (C ₁ to C ₅) and oxygenated
Carbosieves	150	C ₁ to C ₆ compounds
Molecular sieves (5A and 13X)	350	Hydrogen, oxygen, nitrogen, methane and noble gases. Particularly the separation of He/Ne and Ar/O ₂ . Hydrocarbons (C ₁ to C ₃) on 5A with higher alkanes on 13X (up to C ₁₂) but not isomer separations
Cyclodextrins		Fixed gases, halocarbons, hydrofluorocarbons, C ₁ to C ₁₀ hydrocarbons
Porous polymers		
Q	310	Hydrocarbons (C ₁ to C ₁₄), halocarbons (C ₁ and C ₂), volatile
S	250	oxygenated solvents (C ₁ to C ₆), thiols, amines, nitro
U	190	compounds, nitriles, water and inorganic gases

efficiency, are often less favorable for adsorbents compared with liquids, although this is not always the case. Adsorbents are generally more difficult to prepare in a reproducible fashion than liquid phases.

Most interest in gas-solid chromatography today is due to the wider availability and acceptance of porous-layer open tubular (PLOT) columns [128-130]. PLOT columns are more permeable (allowing longer columns to be used), provide sharper peaks, faster separations and quicker column regeneration compared with packed columns. Early versions of PLOT columns contained a layer of physically coated adsorbent particles that lacked adequate stability for general use. Modern PLOT columns are prepared by either *in situ* polymerization or by addition of a chemical binder to the coating solution resulting in immobilization and bonding of particles to the inside column wall. Details of the immobilization methods for commercially available columns are considered proprietary information, about which little is known outside the suppliers laboratory. Yun et al. [131] prepared silica PLOT columns by dynamically coating the inside wall of a fused silica capillary column with a suspension of silica particles containing a poly(methylhydrosiloxane) binder in a volatile solvent. The column was dried and heated to bond the porous silica particles to each other and to the column wall. PLOT columns containing immobilized layers of inorganic oxides, carbon, molecular sieves, cyclodextrins and porous polymers are commercially available in lengths up to 100 m, although shorter lengths are more common, with internal diameters from 0.25 to 0.53 mm and layers of 5 to 50 μ m thick. Typical applications are summarized in Table 2.9. Several columns provide similar separation characteristics of some sample types, but

in general, no single column is able to resolve all members in a family group. Detailed fingerprinting of mixtures usually requires sequential analysis on different column types [128,132].

2.3.6.1 Inorganic oxide adsorbents

The important inorganic oxide adsorbents are silica gel and alumina, both of which have been studied since the early days of gas chromatography [28,109]. These adsorbents are available in a wide range of particle sizes and morphology. For packed column applications rigid spherical beads with surface areas in the range 5-500 m²/g and average pore diameters from 8-150 nm are generally used. Hydrothermal treatment allows the pore diameter of porous silica particles to be adjusted facilitating the preparation of adsorbents with a graded range of properties [133]. Similar porous materials of a smaller average particle size (< 10 µm) are used for the preparation of PLOT columns [128,131,134].

Silica and alumina adsorbents are considered suitable for the separation of low molecular weight saturated and unsaturated hydrocarbons, halogenated hydrocarbons and derivatives of benzene. Polar solutes interact too strongly with these adsorbents to be separated in an acceptable time or with reasonable peak shapes. Dehydrohalogenation and other reactions have been noted for some labile compounds. Retention is a function of the specific surface area, the degree of surface contamination (by water in particular), the prior thermal conditioning of the adsorbent and the ability of the solute to participate in specific interactions, such as hydrogen bonding, with surface functional groups. The different surface functional groups (silanol groups in the case of silica and aluminium ions in the case of alumina) results in different selectivity for these adsorbents. For both adsorbents the uptake of water vapor causes a dramatic reduction in the retention of non-polar compounds. Reproducible separations require the exclusion of moisture from the carrier gas and samples. Mixing the adsorbents with an inert solid diluent, such as glass beads or a diatomaceous support, or coating a support with a layer of fine-particle adsorbent ("dusted columns"), reduces retention and simultaneously improves column efficiency [135]. A more general approach to reduce retention, modify selectivity and/or improve efficiency is to coat the adsorbent with a small quantity of an involatile liquid (usually a common stationary phase) or an inorganic salt [128,136,137]. It is assumed that selective adsorption of the liquid or salt by the most energetic sites, results in a more homogeneous and less retentive surface. At low liquid or salt loadings the retention mechanism is dominated by surface adsorption interactions. At increasing liquid or salt loading gas-liquid or gas-salt adsorption, and eventually gas-liquid partitioning, contribute to retention. Alkali metal salts (potassium chloride and carbonate, sodium sulfate, etc) at loadings of 0.5 to 30 % w/w are common salt modifiers. The optimum salt loading for a particular separation is generally arrived at by trial and error. A typical separation of a mixture of hydrocarbons on a sodium sulfate modified alumina PLOT column is shown in Figure 2.11 [128,138]. In general the unsaturated hydrocarbons are retained longer than the corresponding n-alkanes.

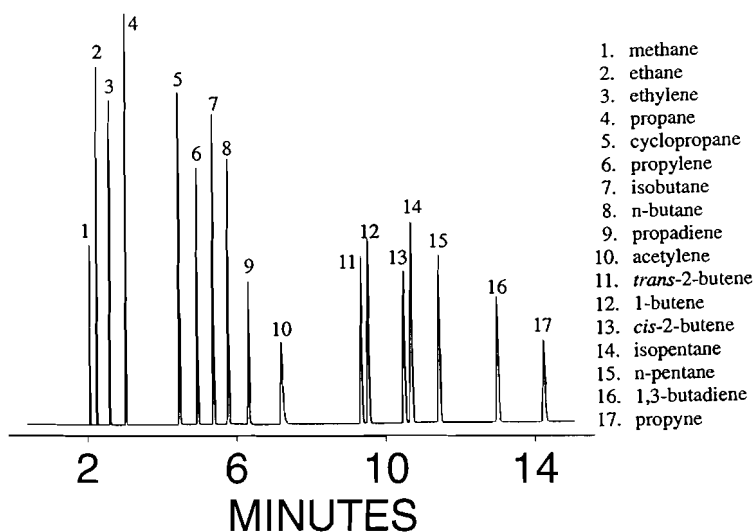


Figure 2.11. Separation of saturated and unsaturated volatile hydrocarbons on a 50 m x 0.53 mm internal diameter sodium sulfate deactivated alumina PLOT column. Carrier gas was helium at 5.0 ml/min and the temperature program 40 to 120°C at 5°C/min with a 5 min hold at 120°C. (©Restek Corporation).

2.3.6.2 Carbon adsorbents

Carbon adsorbents have also been studied since the early days of gas chromatography [139]. Active carbons are rarely used today because of difficulties in standardizing their activity and morphological properties. Graphitized carbon blacks and carbon molecular sieves have superseded them [139-143]. Graphitized carbon blacks are prepared by heating ordinary carbon blacks to about 3000°C in an inert atmosphere. This high temperature treatment destroys functional groups originally present on the carbon surface and induces the growth of graphite crystallites. Graphitized carbon blacks used in gas chromatography are generally nonporous materials with surface areas from about 5 to 100 m²/g. Ideally, they behave as non-specific adsorbents with retention dominated by dispersion interactions with minimal preferential adsorption for compounds containing polar functional groups. In reality, polar adsorption sites, albeit few in number can establish strong specific interactions with polar solutes. High temperature treatment with hydrogen has been used to minimize active sites associated surface oxygen complexes and washing with perchloric or phosphoric acids to remove basic oxygen-containing complexes and sulfur present as sulfide [141]. Even after such treatment, however, further modification of the surface by the addition of small quantities of polar liquids is generally required. At low surface concentrations the liquid modifiers primarily act as tailing inhibitors and retention reducers. Liquids capable of entering into specific interactions with the sample can be used for selectivity adjustment. At surface concentrations of liquid required to build up a densely packed monolayer, retention will occur primarily by adsorption on this layer and the selectivity of the column will be different

to that of a partially complete monolayer. In most cases low liquid concentrations are used to exploit the full advantages of gas-solid chromatographic interactions. Liquid modifiers such as Carbowax 20M, poly(siloxanes), picric acid, etc. (0.2-5 % w/w) are used for packed column separations of a wide range of volatile compounds including alcohols, aldehydes, ketones, terpenes, hydrocarbons and industrial solvents. Addition of phosphoric acid or potassium hydroxide as well as a liquid modifier extends possible applications to organic acids and amines, respectively. Picric acid can be used to improve the separation of saturated and unsaturated hydrocarbons. There were many applications using graphitized carbon blacks and liquid modifiers in the form of efficient micropacked or PLOT columns published in the late 1970s and early 1980s, but this work has not continued into the present day [139,144,145]. PLOT columns prepared from carbon molecular sieves are more widely used today.

Carbon molecular sieves are prepared by the controlled pyrolysis of poly(vinylidene chloride) or sulfonated polymers (Carboxen™) [28,139,145,146]. They consist of very small graphite crystallites crosslinked to yield a disordered cavity-aperture structure. Carbon molecular sieves are microporous and of high surface area, 200-1200 m²/g, with a very pronounced retention of organic compounds. They are used primarily for the separation of inorganic gases, C₁-C₃ hydrocarbons and for the separation of small polar molecules such as water, formaldehyde and hydrogen sulfide. Compounds of higher molecular weight (> C₆) cannot be efficiently desorbed without decomposition of the adsorbent.

2.3.6.3 Molecular sieves

Molecular sieves (zeolites) are artificially prepared alkali metal aluminosilicates. The most common types used for gas chromatography are type 5A, a calcium aluminosilicate with an effective pore diameter of 0.5 nm, and type 13X, a sodium aluminosilicate with an effective pore diameter of 1 nm. The molecular sieves are microporous with a tunnel-like pore structure of similar dimensions to those of small molecules. Retention is primarily governed by the size of the analyte, which determines whether it can enter the pore structure of the molecular sieve, and the strength of adsorption interactions that take place on the internal pore surface and on the particle surface. Molecular sieves are used primarily for the separation of permanent gases (hydrogen, oxygen, nitrogen, carbon monoxide, the inert gases) and low molecular weight linear and branched chain hydrocarbons [26,128,129,147]. Water, carbon dioxide and other polar molecules are retained excessively and catalytic transformations of labile analytes are also common.

2.3.6.4 Porous Polymers

Suspension polymerization, in which a mixture of monomers and crosslinking reagents is polymerized in the presence of an inert solvent, is used to prepare porous polymer beads [148]. The microstructure of the gel, formed within the droplet in the early stages, gradually grows into a sponge-like structure, with inert solvent filling the space between the microstructures. After solvent removal, the porous structure remains, pro-

Table 2.10

Physical properties of porous polymer beads for packed column gas chromatography

STY = styrene; DVB = divinylbenzene; ACN = acrylonitrile; EVB = ethylvinylbenzene; EGDMA = ethylene glycol dimethacrylate; VPO = vinylpyrrolidone; VP=vinylpyridine; DPO = 2,6-diphenyl-p-phenylene oxide

Porous polymer	Monomers	Surface area (m ² /g)	Average pore diameter (nm)	Temperature limit (°C)
Chromosorb 101	STY-DVB	30-40	300-400	275
Chromosorb 102	STY-DVB	300-400	8.5-9.5	250
Chromosorb 103	STY	15-25	300-400	275
Chromosorb 104	ACN-DVB	100-200	60-80	250
Chromosorb 105	Polyaromatic	600-700	40-60	250
Chromosorb 106	STY	700-800	5	225
Chromosorb 107	Acrylic Ester	400-500	8-9	225
Chromosorb 108	Acrylic Ester	100-200	25	225
Porapak N	STY-DVB-VPO	225-500	9	200
Porapak P	STY-DVB-EVB	100-200	7.5-10	250
Porapak Q	EVB-DVB	500-850	7.5-10	250
Porapak R	STY-DVB-VPO	450-600	7.5-10	250
Porapak S	STY-DVB-VP	300-550	7-9	250
Porapak T	EGDMA	250-450	9	200
Porapak PS	Silanized P			
Porapak QS	Silanized Q			250
Tenax-GC	DPO	19-30	25-7500	375

Values for surface area vary widely in the literature

ducing a uniform bead that is sufficiently rigid to be dry packed into a column. For the preparation of PLOT columns suspension polymerization can be performed in the column followed by a second step in which the particles are immobilized onto the inside column wall. Procedural details for the immobilization process are sketchy at best [128,149-152]. By adroit selection of monomer, crosslinking reagent and inert solvent, porous polymer beads of different properties can be prepared, as indicated in Table 2.10. The properties of these sorbents vary with the choice of monomers and the pore structure and surface area of the beads. Porous polymers with average pore diameters less than 10 nm are used primarily for the separation of gases while those with larger pore diameters are used for the separation of volatile and semivolatile organic compounds. Compared to other common sorbents their surfaces are relatively inert and polar compounds such as water, formaldehyde, carboxylic acids and volatile alkylamines, can be separated without appreciable difficulty. Some general guidelines for sorbent selection are summarized in Table 2.11 [153,154] and PLOT column applications are indicated in Table 2.10 [128,152,155]. Differences in monomer ratios and pore structure result in differences in retention properties for notionally equivalent products and between packed and PLOT columns. The sorption properties of the polymers may vary with thermal aging as well.

The retention mechanism for porous polymers is not known exactly. At low temperatures adsorption dominates but at higher temperatures it is possible that the porous polymers behave as a highly extended liquids with solvation properties. Various attempts to characterize selectivity differences based on the system of McReynolds

Table 2.11
Common packed column applications of porous polymers

Polymer	Application
Chromosorb 101 Porapak P and PS	Esters, ethers, ketones, alcohols, hydrocarbons, fatty acids, aldehydes and glycols. Not recommended for amines and anilines.
Chromosorb 102 Porapak Q	Light and permanent gases, volatile carboxylic acids, alcohols, glycols, ketones, hydrocarbons, esters, nitriles and nitroalkanes. Not recommended for amines, anilines. Nitrated by nitrogen oxide gases.
Chromosorb 103 Porapak S	Amines, amides, alcohols, aldehydes, hydrazines and ketones. Not recommended for acids, amines, glycols, and nitriles. Reacts with nitroalkanes.
Chromosorb 104	Nitriles, nitro compounds, sulfur gases, ammonia, carbon dioxide, vinyl chloride, moisture in solvents and xlenols. Not recommended for amines and glycols.
Chromosorb 105 Porapak N	Aqueous mixtures of formaldehyde, acetylene from lower hydrocarbons and most gases. Not recommended for glycols, acids and amines.
Chromosorb 106 Porapak QS	Alcohols, C ₂ -C ₅ carboxylic acids, alcohols and sulfur gases. Not recommended for glycols and amines.
Chromosorb 107 Porapak T	Formaldehyde from water and acetylene from lower hydrocarbons. Sulfur compounds. Not recommended for glycols and amines.
Chromosorb 108	Gases, water, alcohols, aldehydes and glycols.
Porapak R	Esters, ethers, nitriles and nitro compounds. Not recommended for glycols and amines.
Tenax-GC	High boiling polar compounds, diols, phenols, methyl esters of dicarboxylic acids, amines, diamines, ethanolamines, amides, aldehydes and ketones.

phase constants, Snyder's selectivity triangle, the difference in apparent carbon number for n-alkanes and n-alcohols and the relative retention of ethylene, acetylene and carbon dioxide have been proposed, but the outcome is not very satisfying [154,156,157].

With the exception of Tenax-GC, the porous polymers are limited in application to those compounds with boiling points less than about 300°C; otherwise, retention times may be excessively long. Tenax-GC is a linear polymer of p-2,6-diphenylphenylene oxide with a molecular weight of about 5×10^5 to 10^6 . It differs from the other porous polymers in that it is a granulated powder of low surface area. Its separation characteristics are similar to the EDB-DVB porous polymers, except for its higher thermal stability, which allow its use at isothermal temperatures up to 375°C.

2.3.7 Miscellaneous Materials

The continuous evolution of stationary phase technology results in the introduction of new materials and the phasing out of older materials considered obsolete. For

completeness we will consider three groups of materials representing examples that could fit into either of these categories.

2.3.7.1 Inclusion formation

Inclusion is a specific spatial interaction in which the molecule of a guest (or at least part of it) is included in the cavity of the host, and retention is influenced by the characteristic properties of the host-guest interactions. Suitable host molecules include cyclodextrins, calixarenes and resorcarenes. The best known example is the cyclodextrins, which have been exploited in several chromatographic techniques, for the separation of isomers and enantiomers (see section 8.3.6 and 10.4.1). Cyclodextrins are cyclic oligosaccharides containing 6, 7, or 8 glucose units (designated α -, β -, γ -cyclodextrin, respectively) with the shape of a hollow truncated cone of dimensions determined by the number of glucose units. The cavity is relatively non-polar while the outer surface and the rim is substituted with hydroxyl groups. Native cyclodextrins have high melting points and have been used in gas-solid chromatography to separate a wide range of volatile organic compounds with properties complementary to those of inorganic oxide and carbon adsorbents, Table 2.9 [158-160]. Cyclodextrins are thought to retain analytes by two distinct mechanisms as a consequence of the difference in polarity of their interior and exterior surfaces and the size selectivity represented by the dimensions of the cavity. Polar compounds have the capability to form hydrogen bonds and dipole-type interactions with the hydroxyl groups on the outer surface of the cavity whereas selectivity for non-polar compounds depends on their fit within the cavity.

Calixarenes [161-166] and resorcarenes [166,167] are emerging materials with properties expected to be complementary to those of the cyclodextrins, but have yet to find any useful applications in gas chromatography. The calixarenes are macrocyclic phenol-formaldehyde polycondensates possessing basket-shaped intramolecular cavities. Native calixarenes are insoluble in common solvents and have poor coating characteristics resulting in low column efficiency. The introduction of non-polar substituent groups resulted in new materials soluble in the poly(methylphenylsiloxane) solvent, OV-1701. Columns coated with dissolved calixarenes or calixarene-containing poly(siloxanes) exhibited favorable chromatographic properties as well as providing some evidence for the formation of inclusion complexes with simple aromatic compounds.

2.3.7.2 Bonded sorbents

Bonded phases were developed to provide column packings with high efficiency and low column bleed compared with coated liquid phases. Early developments included the development of estersils (DurapakTM) prepared by the condensation of an alcohol or isocyanate reagent with porous silica beads [168,169]. Bonded groups included octane (by reaction with octanol), phenyl, propionitrile and Carbowax. Only a monomeric film is possible due to the specific nature of the reaction. Favorable mass transfer properties allow high flow rates to be used for fast separations while retaining reasonable column efficiency. Retention depends on the size and functionality of the organic chain, the degree of surface bonding, and the size and polarity of the solute. For short chain bonded

groups, retention is dominated by adsorption on either the silica support surface or the bonded organic group. For phases containing polar groups, adsorption at the surface of the bonded organic group tends to dominate the retention mechanism. Long chain bonded alkyl groups show less spatial ordering than their short chain analogs, and the possibility that partitioning contributes to the retention mechanism is more likely. The bonded phases are not very stable, however, and are easily hydrolyzed by water and other proton-donor substances.

More stable bonded phases can be prepared by the reaction of a monofunctional or multifunctional alkylsilane or cyclosiloxane reagent with a porous silica support or an alkene with a hydrosilane modified silica support [170,171]. In this way, alkane groups of different chain length, squalane and phenyl groups have been bonded through siloxane groups to the silica surface resulting in sorbents suitable for the separation of low molecular weight hydrocarbons in short micropacked columns.

The high-temperature treatment of a liquid-coated diatomaceous support under a slow flow of gas followed by exhaustive solvent extraction results in the bonding of a small quantity ($\approx 0.2\%$ w/w) of the original liquid phase on the support surface (Ultrapond™)[172-174]. Liquid phases bonded in this way include Carbowax 20M, poly(esters) and poly(siloxanes). The retention mechanism on these nonextractable phases is poorly understood, but it is believed that they probably act as adsorbents, similar to the estersils, except that the liquid film may be present, in part, as "dense patches" and, therefore a greater contribution from partitioning is anticipated. Compared with conventionally coated phases these sorbents are characterized by low retention, high efficiency, very low column bleed and a higher maximum operating temperature.

Polymer encapsulated sorbents have been prepared by peroxide-induced or ozone-induced free radical crosslinking of coated porous silica or diatomaceous supports at elevated temperatures [175]. By reloading and performing sequential crosslinking reactions up to 20% (w/w) of liquid phase can be immobilized over the support surface. This reaction is analogous to the widely used procedure for immobilizing stationary phases in open tubular columns. Few practical applications have been demonstrated.

2.3.7.3 *Metal-containing sorbents*

Complexation chromatography allows the separation of electron donor compounds by exploiting the fast and reversible equilibrium between suitable solutes and an electronically and coordinatively unsaturated metal. These interactions are sensitive to subtle differences in the composition and stereochemistry of the donor compound since the strength of the donor-acceptor bond is influenced by electronic, steric and strain effects (section 10.6.3). Poly(methylsiloxanes) containing 3-cyanopropyl and 3-mercaptopropyl substituents are suitable for bonding transition metals (copper, nickel, cobalt) for preparing immobilized stationary phases in open tubular columns [176]. Porous silica sorbents with siloxane bonded 3-aminopropyl, N-(2-aminoethyl)-3-aminopropyl and iminoketone groups have been used to complex copper and chromium [177,178]. These approaches result in thermally stable and efficient columns. Retention

for a wide range of compounds on the metal-containing and metal-free materials show only small differences suggesting limited practical possibilities for controlling selectivity.

Metal (II) bis[(3-heptafluorobutonyl)-(1R)-camphorates] containing manganese, cobalt and nickel dissolved in a noncoordinating stationary phase (e.g. 0.1 molal metal complex in a poly(dimethylsiloxane) solvent), or as an immobilized film of a poly(methylsiloxane) containing metal bis[(3-heptafluorobutanoyl)-10-methylene-(1R)-camphorate] substituents on open tubular columns for the separation of isotopomers, isomers and enantiomers of hydrocarbons and oxygen, nitrogen and sulfur-containing electron-donor solutes (section 10.6.3) [179,180]. Examples include pheromones, cyclic ethers, 1-chloroaziridines, thiranes, thiethanes, acetals, esters, ketones and alcohols sufficiently volatile to be separated at temperatures less than about 100° for the dissolved metal complexes and less than about 150°C for the immobilized metal-complex stationary phases. Although camphorates have been the most widely used complexing ligands useful properties for the separation of enantiomers have been observed for a number of terpeneketonates (e.g. carvone, menthone, 4-pinane). Metal complex coated capillary columns are deteriorated by hydrogen carrier gas, which should be avoided. Kowalski [118] characterized the retention properties of a series of lanthanide tris[3-(trifluoromethylhydroxymethylene)camphorate] complexes dissolved in a poly(dimethylsiloxane) stationary phase. The lanthanide chelates are “hard acids” and selectively retain “hard bases” (e.g. alcohols) and to a lesser extent “soft bases” such as ketones, ethers, and aromatic and olefinic compounds. With decreasing metal ionic radius the lanthanide chelates change from (relatively) “soft” to “hard” according to Pearson’s principle allowing adjustment of selectivity by using different metals. Despite extensive research metal complex stationary phases have found few practical applications with the exception of the metal camphorates used largely for the separation of enantiomers (section 10.6.3).

2.4 RETENTION IN GAS-LIQUID CHROMATOGRAPHY

Early attempts to understand retention in gas-liquid chromatography were based on a partition model. Partitioning is generally the dominant contribution to retention for all compounds at intermediate temperatures. However, any refined approach to understanding retention must also take into account contributions from interfacial adsorption and interactions with the structured liquid film in immediate contact with the support surface [181]. Interfacial adsorption includes interactions at the gas-solid (support) interface and gas-liquid interface. Interfacial adsorption by the support usually result from polar interactions between the solute and support functional groups, typically silanol groups, which are inadequately masked by the deactivation steps during column preparation. Interfacial adsorption at the liquid interface is generally observed for solutes with limited solubility in the bulk liquid stationary phase, and is most important for non-polar solutes on polar stationary phases. It is generally

more significant for all types of compounds on highly cohesive stationary phases and makes a greater contribution to the retention mechanism at low phase loadings due to a combination of a larger accessible liquid surface area and a smaller bulk liquid volume. When the retention mechanism is characterized as a mixed retention process interfacial adsorption increases in importance at lower temperatures.

Any model devised to explain retention in general terms must also take into account the distribution of the liquid phase on a porous surface [44,182]. For liquids that do not wet the support readily at low phase loadings, the liquid phase will be present as droplets or pools of liquid located primarily at the outer grain surface with little penetration into the pores. At higher phase loadings coalescence from microdroplets to a continuous film will occur. Prior to coalescence the solute is exposed to a large support area and a very small liquid area, a situation that is reversed after coalescence. For liquids with good support wetting characteristics it is generally assumed that at low loadings the liquid phase is first adsorbed as a monomolecular and multimolecular layer over the entire support surface. As the phase loading is increased it collects, initially, in the fine pores first, and then progressively appears in large cavities at the same time as the adsorbed layer thickens up. For diatomaceous earth supports with typical surface areas from 1-3 m²/g a 0.01 to 2% (w/w) liquid phase loading should be adequate to cover the support surface with a monolayer, depending on the orientation of the adsorbed molecules. Liquid phase molecules near the support surface exist in a regular arrangement with entropy of solution that is lower than that of the bulk liquid. It is unlikely that surface influences become completely non-existent beyond the first monolayer or so, for although the forces themselves are of very short range, they can be transmitted by successive polarization of adjacent molecules to a considerable depth in the liquid. The propagation of these forces is opposed by the thermal motion of the molecules and so falls off with distance after the strongly attracted first monolayer. The structured layer, therefore, may be of considerable thickness and will likely dominate the retention characteristics of the liquid phase at low phase loadings. The characteristic properties of the liquid surface will be different for structured and bulk liquid films. The extent of the liquid surface area will change in a nonlinear manner with increasing phase loading until, at high phase loadings, it asymptotically approaches a limiting value, approximately equivalent to the support surface area less the area of its narrow pores and channels [183].

2.4.1 General Model

Taking the above considerations into account, a general model for retention in gas-liquid chromatography is given by Eq (2.2) [29,184-188]

$$V_N^* = V_L K_L + \delta V_L (K_S - K_L) + (1 - \delta) A_{LS} K_{DSL} + A_{GL} K_{GL} + A_{LS} K_{GLS} \quad (2.2)$$

where V_N^* is the net retention volume per gram of packing, V_L the volume of liquid phase per gram of packing, K_L the gas-liquid partition coefficient, δ a constant

Table 2.12

Relative contribution (%) of different retention mechanisms for different solutes on Apiezon M and Carbowax 4000 based on Eq. (2.2) at 70°C.

$V_S K_S$ = contribution due to partition with the structured liquid phase layer

Solute	Phase	Interaction	Phase Loading (%)			
			1	4.7	9.1	17
Octane	Apiezon	$V_L K_L$	100	100	100	100
	Carbowax	$V_L K_L$	58	88	93	97
		$A_{GL} K_{GL}$	42	12	7	3
Benzene	Apiezon	$V_L K_L$	100	100	100	100
	Carbowax	$V_S K_S$	89	98	56	28.2
		$V_L K_L$			43	71.3
Chlorobenzene	Apiezon	$A_{GL} K_{GL}$	11	2	1	0.5
		$V_L K_L$	100	100	100	100
		$V_S K_S$	96	99	62.8	31.4
	Carbowax	$V_L K_L$			36.8	68.4
		$A_{GL} K_{GL}$	4	1	0.4	0.2
2-Butanone	Apiezon	$V_L K_L$	52	84	92	96
		$A_{LS} K_{GLS}$	48	16	8	4
		$V_S K_S$	82	96	60	30
	Carbowax	$V_L K_L$			38	69
		$A_{GL} K_{GL}$	18	4	2	1
1-Butanol	Apiezon	$V_L K_L$	13	42	67	75
		$A_{LS} K_{GLS}$	87	58	33	25
		$V_S K_S$	86	97	60	30
	Carbowax	$V_L K_L$			38	69
		$A_{GL} K_{GL}$	14	3	2	1

constrained to have values of 1 when the film thickness is less than or equal to the thickness of the structured layer (d_S) and zero when the film thickness exceeds the thickness of the structured layer, K_S the gas-liquid partition coefficient for the structured liquid layer, A_{LS} the liquid-solid interfacial area per gram of packing, $K_{DSL} = d_S(K_S - K_L)$, A_{GL} the gas-liquid interfacial area per gram of packing, K_{GL} the adsorption coefficient at the gas-liquid interface, A_{LS} the liquid-solid interfacial area per gram of packing and K_{GLS} the coefficient for adsorption at the liquid-solid interface. Although Eq. (2.2) provides a general description of the retention process it is rather awkward to use. Eq. (2.2) contains five unknowns (K_L , K_S , K_{DSL} , K_{GL} , and K_{GLS}) which requires data for the packing characteristics (V_L , A_{GL} , and A_{LS}) for at least five column packings prepared from the same support at different phase loadings to obtain a numerical solution. In addition, there is no exact method of defining the thickness of the structured layer, which must be established by a trial and error procedure. Nikolov [187] applied Eq. (2.2) to the system Apiezon M and Carbowax 4000 on Celite at phase loadings of 1-18% (w/w). An abbreviated summary of Nikolov's data is presented in Table 2.12. Gas-solid adsorption of polar solutes contributes substantially to retention on the Apiezon columns. Even at high liquid phase loadings, adsorption at the support surface may contribute between 4 and 25% of solute retention. For Carbowax 4000, at low phase loadings adsorption at the gas-liquid interface may contribute between 3 and 42% to the

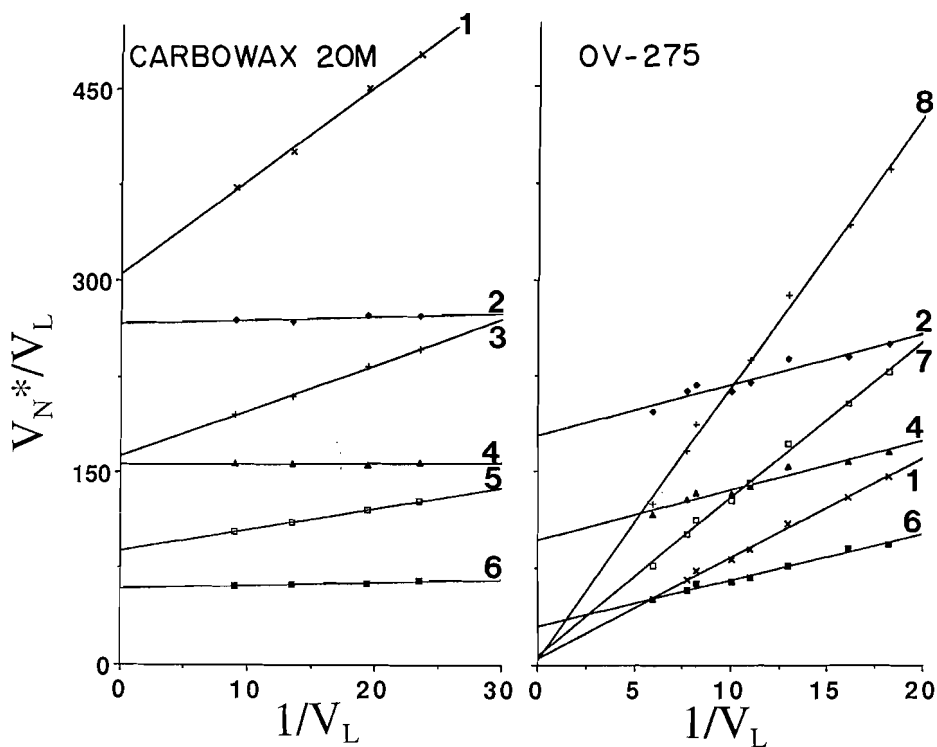


Figure 2.12. A plot of V_N^* / V_L against $1 / V_L$ for several compounds on Carbowax 20M and the poly(dicyanoalkylsiloxane) OV-275 on Chromosorb W-AW at 80.8°C. Identification; 1 = dodecane; 2 = nitromethane; 3 = undecane; 4 = dioxane; 5 = decane; 6 = ethanol; 7 = tridecane and 8 = tetradecane.

retention of the solutes investigated while at high phase loadings its relative contribution is small.

By working at intermediate to high phase loadings the contributions of the structured liquid phase to retention can be neglected allowing Eq. (2.2) to be simplified and rearranged to

$$V_N^* / V_L = K_L + (A_{GL}K_{GL} + A_{LS}K_{GLS})(1/V_L) \quad (2.3)$$

from which the gas-liquid partition coefficient can be determined from a plot of V_N^* / V_L against $1/V_L$ for a series of column packings by extrapolation [29,81,188-190]. The shape of the plot is dependent on the relative importance of adsorption at the gas-liquid and gas-solid interfaces. Generally, one of these terms will tend to dominate and in most cases a linear extrapolation can be used. Some representative examples are shown in Figure 2.12 for n-alkanes, nitromethane, dioxane and ethanol on the polar stationary phases Carbowax 20 M and the poly(dicyanoalkylsiloxane)

OV-275. For solutes retained solely by gas-liquid partitioning (e.g. nitromethane, dioxane and ethanol) on Carbowax 20M the plots have a zero slope. The n-alkanes are retained by a mixed retention mechanism on Carbowax 20M as indicated by the slope. The relatively large intercept is an indication that gas-liquid partitioning makes a significant contribution to the retention mechanism. Interfacial adsorption is important for all compounds on OV-275 and is dominant for the n-alkanes, which have a near zero intercept, indicating that gas-liquid partitioning is of minor importance to their retention. The lack of a reliable method to estimate the surface area of the liquid phase prevents Eq. (2.3) from being used to determine the gas-liquid adsorption coefficient.

The Δ function, defined by Eq. (2.4), provides a semi-quantitative index of the relative contribution of interfacial adsorption to retention on a packed column:

$$\Delta(\%) = 100[\log K - \log K_L] / \log K_L \quad (2.4)$$

where K is the observed distribution constant for sorption of a solute in a particular chromatographic system. When retention occurs exclusively by gas-liquid partitioning, $\log K = \log K_L$ and $\Delta = 0\%$. For a varied group of solutes on poly(ethylene glycol succinate) Δ values of 0-35 % [192], on Carbowax 20M Δ values of 0-10 % [123] and on squalane Δ values of 0-12 % [43] were observed for stationary phase loadings between 8 and 20 % (w/w) and temperatures within the range 60-140°C. Whether $\log K$ or $\log K_L$ is used as the dependent variable in the solvation parameter model (section 1.4.3 and 2.3.5) it is usually possible to obtain acceptable models but the system constants are different when interfacial adsorption is an important retention mechanism and usually the uncertainty in the sorption model is greater. These differences are important in mechanistic studies, the simulation of retention and the estimation of solute descriptor values [104,192]. As a unifying framework it was suggested that the retention properties of the interfacial region resemble those of the bulk phase except that the interfacial region is less cohesive and the intensity of polar interactions are reduced.

There are certain conditions that must be fulfilled if Eqs. (2.2), (2.3) and (2.4) are to be used to calculate partition coefficients. The basic assumption is that the individual retention mechanisms are independent and additive. This will be true for conditions where the infinite dilution and zero surface coverage approximations apply or, alternatively, at a constant concentration with respect to the ratio of sample size to amount of liquid phase. The infinite dilution and zero surface coverage approximations will apply to small samples where the linearity of the various adsorption and partition isotherms is unperturbed and solute-solute interactions are negligible. The constancy of the solute retention volume with variation of the sample size for low sample amounts and the propagation of symmetrical peaks is a reasonable indication that the above conditions have been met. For asymmetric peaks, however, the constant concentration method must be employed if reliable gas-liquid partition coefficients are to be obtained [191]. It is difficult to state absolutely the conditions for which contributions to retention from the structured liquid phase layer can be neglected. This will occur for some minimum phase loading that depends on the support surface area, the liquid phase

support wetting characteristics and possibly the column temperature. Occasionally this will be true for phase loadings below 5% (w/w) on typical diatomaceous earth supports but is more likely to be generally true at phase loadings exceeding 10% (w/w).

There are fewer studies on the retention mechanism for open tubular columns but the theory presented for packed columns should be equally applicable. For films of normal thickness open tubular columns have a large surface area/volume ratio and the contribution of interfacial adsorption to retention will be significant for those solutes with limited solubility in the stationary phase [42]. Interfacial adsorption was shown to affect the retention reproducibility of polar solutes on non-polar stationary phases of different film thicknesses and the retention index values of solutes on polar stationary phases due to liquid phase interfacial adsorption of the n-alkane retention index standards [184,188,193-197].

2.4.2 Binary Stationary Phase Mixtures

Consider the situation where a sample is incompletely separated on two stationary phases and the incompletely separated components are different on either stationary phase. There must be a good chance that a complete separation could be obtained by combining the two stationary phases in the correct proportions. If this could be predicted from the data already collected on the two single stationary phases, this would be all for the better. Before presenting the theory to do this, we should note the different options available for combining the stationary phases. A support could be coated with an optimized mixture of two stationary phases (mixed solvents); two column packings, each coated with a single stationary phase, could be mixed in the optimum proportions for the separation (mixed bed); or two columns containing different stationary phases could be coupled together (serially coupled or tandem columns). For packed columns, the mixed bed approach is the most versatile and economical and results in identical separations to the mixed solvent approach in the absence of specific interactions between phases that are not accounted for by the theory [198]. For open tubular columns the serial coupling of columns is the most versatile and economical approach and provides similar separations to columns coated with mixed solvents [199,200]. It can be difficult to prepare open tubular columns with mixed stationary phases due to miscibility problems and the difficulty of finding a single deactivation procedure compatible with the requirements of both phases. Some mixed solvent columns designed for specific, often regulatory applications, are available, although even here, it is preferable to design a single-composition stationary phase based on the optimized composition of the mixed phases [201,202]. In this case the optimization strategy is an approach to the rationale design of new stationary phases with properties tailored for particular applications. This is reasonably straightforward when the single phases are poly(siloxanes) containing different substituents and the new phase is a poly(siloxane) prepared from the predicted proportions of the appropriate monomers.

Purnell and coworkers [198,203,204] developed the general theory for retention on mixed phases based on the simple relationship, $K_S = \phi_A K_A + \phi_B K_B$, where K_S is the

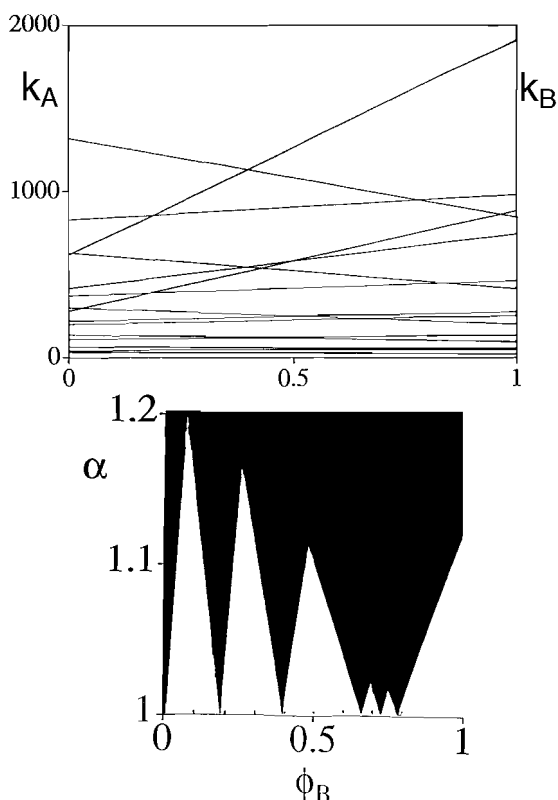


Figure 2.13. Plot of the gas-liquid partition coefficients (K_A and K_B) on two stationary phases to estimate the gas-liquid partition coefficients for all solutes at any intermediate mixed-phase composition and the resulting window diagram plot of the separation factor (α) as a function of the volume fraction of the B stationary phase. (From ref. [205];©Elsevier)

gas-liquid partition coefficient for a solute on the mixed bed or mixed solvent column, K_A and K_B are the gas-liquid partition coefficients for the solute on the single stationary phases A and B, and ϕ_A and ϕ_B are the volume fractions of stationary phase. Since $\phi_A + \phi_B = 1$, the above relationship can be rearranged to express the gas-liquid partition coefficient for a solute on the mixed phase as a linear function of the volume fraction of one of the phases and the difference in gas-liquid partition coefficients for the solute on the single phases, $K_S = K_A + \phi_B(K_B - K_A)$. A plot of K_S against ϕ_B is a series of straight lines of slope determined by the single-phase partition coefficients plotted as the extreme points on the dual ordinate axis, Figure 2.13 [205]. The wide range of slopes and the numerous crossover points is an indication of the potential of mixtures of the two phases to influence relative retention. The elution order of the solutes at any binary phase composition can be read by constructing a vertical line at the composition of

interest. For identification of the optimum separation conditions, Purnell and coworkers [205-207] introduced the window diagram approach.

The window diagram is a plot of the separation factor for all solute pairs as a function of the volume fraction of one of the liquid phases, Figure 2.13 [205]. The separation factor is simply the ratio of the gas-liquid partition coefficients determined at any phase composition from plots similar to Figure 2.13. To simplify the calculation all solute pairs with a separation factor greater than 1.2 are ignored, since these separations are trivial. Also, for the purpose of visualizing the data, when plots of K_S against ϕ_B cross for any solute, the calculated value of the separation factor is inverted so that the separation factor is ≥ 1 at all times. The resulting window diagram is a series of approximate triangles rising from the volume fraction axis that constitute the windows within which complete separation for all solutes can be achieved. From the window diagram it is immediately obvious whether a separation of all components is possible together with an indication of the anticipated difficulty of performing the separation for the optimum or other binary mixture compositions. The optimum stationary phase composition for the separation corresponds to the value of ϕ_B for the highest window provided that the most difficult to separate pair are reasonably well retained. When the most difficult pair to separate are weakly retained a different strategy may be required [207,208]. In addition to the optimal binary stationary phase composition and the elution order, the window diagram method can also predict the required column length and the total separation time. Since the separation and retention factors are identified during window diagram optimization the plate number required for a selected resolution can be estimated from the resolution equation (section 1.6.1). The column plate height for the separation is estimated from the measurements made on the single-phase columns by assuming that the mixed phase exhibits the same efficiency. The column length necessary to give the required plate number can then be calculated. Once the column length is established the separation time can be calculated for the most retained component of the mixture. When separation speed is the main consideration a smaller window may be an acceptable compromise in which some loss in resolution for the critical pair is given up in favor of lower retention for the most retained component [208]. If the number and identity of the sample components are unknown, it is possible to optimize the separation by trial separations on the single phases and three binary phases with volume fraction ratios of 2:1, 1:1 and 1:2 [209]. Since for each solute the plot of the gas-liquid partition coefficient against the volume fraction of one phase must lie on a straight line, a plot similar to Figure 2.13 is easily constructed and a window diagram plot derived in the usual way. To further advance the use of the window diagram optimization method computer programs have been published for constructing the window diagrams [210] and modifications to the theory made to allow the use of other retention properties such as the retention factor, specific retention volume or retention index [203,211,212]. The above theory assumes a partitioning model for the retention mechanism and predictions will be approximate or poor for solutes retained by a mixed retention mechanism involving interfacial adsorption (section 2.4.1).

Table 2.13

Summary of relationships used to optimize separations by serially coupled columns

Subscript s is for the system and 1 for the first (front) and 2 for the second (rear) column. A * indicates a column parameter normalized per unit length. L is the column length, V_M the hold-up volume, p_i inlet pressure for column 1, p_o the outlet pressure for column 2, and u_o the column outlet velocity.

Parameter	Relationship
Retention factor	$k_S = (Pk_1 + k_2) / (P + 1)$ $P = (t_{M1} / t_{M2})$ $k_S = f_1 k_1 + f_2 k_2$
Ratio of column hold-up times	$P = (L_2 R_2 / L_1 R_1)(V_{M1} / V_{M2})^2 [(p_i^3 - p_o^3) / (p^3 - p_o^3)]$
Resistance to flow	$R = (p_i^2 - p_o^2) / p_o u_o$
Junction pressure	$p^2 = (p_i^2 - f_1 [p_i^2 - (V_{M2} R_1 / V_{M1} R_2) p_o^2]) / (1 - f_1 [1 - (V_{M2} R_1 / V_{M1} R_2)])$ $p^3 = (p_i^3 - f_1 (p_i^3 - \gamma p_o^3)) / (1 - f_1 [1 - \gamma])$
Length fraction	$f_1 = L_1 / (L_1 + L_2)$ $f_1^{-1} = ([R_1 * V_{M2} * / R_2 * V_{M1} *] [(p^2 - p_o^2) / \{p_i^2 - p^2\}] + 1)$
Normalization factor	$\gamma = ([R_1 / R_2][V_{M2} * / V_{M1} *]^2)$
Linearization function	$f = f_1 + f_2$ $f = P / (P + 1)$

Unlike mixed bed columns the overall retention on serially coupled columns is not a simple average of the individual column values due to the influence of gas compressibility on retention and, further, depends on the column sequence. A comprehensive theory for system optimization of serially coupled packed or open tubular columns was proposed by Purnell and coworkers [204,213,214], extended, and validated by others [199,215-223]. The necessary equations are summarized in Table 2.13. Application of the theory requires calculation of the resistance to flow for the individual columns. This is obtained from a plot of the column hold-up time as a function of the inlet pressure for each column. Retention factors for the mixture components are measured for each column and the column hold-up volume is calculated. The two columns can then be coupled fixing the length fraction, the inlet pressure set to provide an acceptable plate height, and the outlet pressure for the column tandem measured. Theory then allows optimization of the system retention factor as a function of the column junction pressure. Alternatively, the optimum column lengths for a separation can be calculated using the window diagram approach. The separation factor is calculated for all solute pairs in the mixture as a function of f_1 , the linearization function (Table 2.13). The window diagram is plotted for the separation factor as a function of f_1 ($0 \rightarrow 1$) ignoring values of $\alpha > 1.1$ since separation factors of this magnitude represent trivial separations on open tubular columns. From the highest window the maximum value of α for separation of the critical pair and the retention factor are obtained from which the resolution equation allows the required plate number

for the separation to be calculated. From the average value of the plate height on both columns the total column length is calculated from the estimated required plate number and the individual column lengths from the total column length and the calculated value for the length fraction. The calculations are algebraically complex but are easily accomplished with a spread sheet program.

In practice, the residence time of the sample in the two columns can be adjusted by changing the relative column lengths, by adjusting the carrier gas velocity in the two columns to selected values, or by operating the columns at different temperatures in a dual oven gas chromatograph. For convenience, the column junction is both pressure and flow controlled and may allow the possibility of diverting a fraction of the sample from the first column to a detector to aid accurate setting of the operating conditions for the first column [200,218,224]. The residence time of the sample in the first column of the tandem will depend on the pressure drop between the column inlet and the coupling point (junction). For the second column the residence time depends on the pressure drop between the coupling point and the outlet for the column tandem, which will normally be atmospheric when the flame ionization detector is used. It is also possible to have a higher or lower flow in the second column compared to the first by adding or releasing carrier gas at the coupling point. The theory was developed for isothermal separations, but in an approximate form, has been extended to simultaneous temperature program operation of both columns to minimize the separation time for mixtures of wide volatility [221,223-225]. Additional isothermal experiments are required to establish the relationship between program rate and retention and the agreement between predicted and experimental results is generally only fair.

At present neither mixed bed packings nor serially coupled columns are widely used outside of a few research centers. A number of factors probably contribute to this. The high efficiency of open tubular columns enables many separations to be performed on columns with adequate but not optimum selectivity. The theory may seem forbidding to some analysts and the absolute control of pressure prior to the introduction of electronic pressure control was inadequate to provide reproducible retention for serially coupled columns. Unless a large number of identical samples require analysis it will be difficult to recover the time required for system optimization. Column deterioration due to thermal aging or sample contamination may require periodic system reoptimization. Pressure control with fixed length columns is the most versatile approach if different samples are to be separated but is not as powerful as column length optimization. This is because a minimum carrier gas flow must be provided to maintain a useful separation efficiency and together with the limited range of possible junction pressures means that selectivity adjustment is restricted to a range less than the selectivity extreme of the two columns. The use of serially coupled columns in fast gas chromatography and process applications represents growth areas at present.

2.4.3 Temperature and Flow Program Operation

Temperature program and isothermal operation are complementary separation techniques in gas chromatography. Temperature program operation is used for the separation

of mixtures with a wide volatility range that cannot be separated in an acceptable time at a constant temperature (section 1.4.5). In addition, temperature program operation is the most useful approach for estimating the complexity of unknown samples and is compatible with large volume injection techniques for trace analysis employing cold trapping. Temperature program separations are performed under conditions of constant pressure or constant flow. Constant mass flow through the column is achieved by the simultaneous increase in the column inlet pressure with increasing temperature to compensate for the change in carrier gas viscosity. Although all manner of claims are made in the literature, it is likely that separation times are significantly different for columns operated at their highest peak capacity in the constant pressure or constant flow modes [226]. Constant flow operation is preferable for separations recorded with flow sensitive detectors (e.g. mass spectrometers). For the same temperature program and different inlet pressures or operating modes (constant pressure or constant flow) changes in elution order are sometimes observed but are not easily predicted without resort to detailed computer simulation models of the separation process [226,227]. Different temperature program rates can also result in changes in elution order [229]. Consequently, the program rate has to be considered an important variable in selectivity optimization.

Flow programming can achieve similar results to temperature programming, although the change in selectivity that accompanies variation in temperature will likely be different to that caused by changes in flow. In practice, temperature program operation is the more important separation technique, with flow program operation reserved for special applications, particularly in process analysis [214]. Flow program operation shortens the separation time for mixtures of wide volatility, while permitting column operation at a lower temperature. The separation of thermally labile samples may be facilitated, column bleed is reduced, and since pressure changes are virtually instantaneous, the cycle time to the start of the next separation is shorter. Disadvantages of flow program operation include decreased efficiency for late eluting peaks, problems with calibrating flow sensitive detectors, and the inconvenience of high-pressure operation. Exponential changes in flow are required for fast separations limiting applications to short or wide-bore open tubular columns [230].

A typical temperature program consists of a series of controlled changes in the oven temperature. Most programs are simple, consisting of an initial isothermal period, a linear temperature rise segment, a final isothermal period at the terminal temperature of the rise segment, and a cool down period to return the oven to the starting temperature. The initial and final isothermal periods are optional, the temperature rise segment can be selected over a wide range (0.1 to ca. 50°C / min), and for complex mixtures, several sequential linear programs and isothermal segments can be used to optimize the separation. Non-linear temperature programs are rarely used. The initial oven temperature is selected with due consideration to the resolution of the earliest eluting peaks in the chromatogram. If the temperature chosen is too high the resolution of the initial peaks may be inadequate, and if it is too low, resolution may be acceptable but the separation time will be extended needlessly. The final temperature should be selected so that the termination of the temperature rise segment and elution of the last

sample component coincide unless the last few eluting peaks are particularly difficult to separate and require isothermal conditions. The selection of the program rate represents a compromise between the necessity of maintaining a minimum acceptable resolution for the sample and the desire to reduce the separation time. This is governed largely by the complexity of the sample and its volatility range. Slow program rates are generally required for difficult separations. Carrier gas flow rate rather than program rate is usually adjusted to optimize the separation time of weakly retained solutes. At a constant flow rate increasing the program rate causes a proportional decrease in the separation time for strongly retained solutes. Software has been developed for the constant pressure mode that allows scaling of the temperature program rate to preserve the peak elution pattern for separations using open tubular columns coated with the same stationary phase but with different dimensions, outlet pressures, and carrier gases [231,232]. A general optimum program rate of ca. $10^{\circ}\text{C} / t_{\text{M}}$ is suggested for the separation of a broad range of compounds by gas-liquid partition chromatography [232,233]. The rapid baseline rise associated with stationary phase bleed in packed column gas chromatography can be highly attenuated by using identical dual columns and simultaneous detection in the difference mode.

A rigorous theoretical treatment of temperature-program gas chromatography is not straightforward [232-242]. The fundamental equation for linear temperature programs is $dt / t_{\text{R}} = dl / L$ where L is the column length, dl the distance traveled by the solute in time dt , and t_{R} is the isothermal retention time for the same solute at temperature T . Unfortunately, this integral has no analytical solution and either numerical methods or simplifying approximations must be used. Computer simulations are fast and practical but the underlying processes are not intuitively informative. Computer simulations, therefore, are more relevant for optimization than for comprehension of the separation process.

It is convenient to divide computer simulation methods into those employing isothermal retention data or retention indices to predict temperature program retention times and those using a small number of temperature program separations to predict retention times for other program conditions. In the former case two or three separations are performed at different isothermal temperatures to estimate appropriate values for the phase ratio and the standard enthalpy and entropy of transfer from the gas phase to the stationary phase for each solute (see van't Hoff plot in section 1.4.4). The recovery of thermodynamic data from retention indices requires that the adjusted retention time for two *n*-alkanes at the same temperature on the column used in the simulation are available [240]. To predict program retention times the column hold-up time and its variation with operating conditions is required. This is obtained from direct measurements on the column to be used for the separation or by calculation of an effective column hold-up time by considering the effect of all temperature-dependent parameters and their changes during the program segments [239]. The temperature program retention times for each solute are then obtained by summation of the retention factors calculated from the thermodynamic data for all segments in the program and the effective column hold-up time. Alternatively, the program retention times can be determined by dividing the chromatographic process into a series of short segments

Table 2.14

Relationships for predicting retention in temperature program separations using the linear-elution-strength model.

T_f = final program temperature, T_o = initial program temperature, t = time, t_p = program time for a linear temperature program, t_M = column hold-up time, k_o = initial value of the retention factor at the start of the temperature program, w_b = peak width at base and N = column plate count.

Parameter	Relationship
Retention time (linear program)	$t_R = (t_M / 2.3b) \ln [e^{2.3b}(k_o + 1) - k_o]$
Fractional migration of solute (segmented programs)	$r = (1 / 2.3b) \ln [(e^{2.3bt_M} + k_o) / (k_o + 1)]$
Retention factor	$\log k = A' - ST$
Program-steepness parameter	$b = t_M(T_f - T_o)S / t_p$
Bandwidths	$w_b = 4t_M(1 + k) / \sqrt{N}$

corresponding to very small time intervals within which both the retention factor and the carrier gas velocity are assumed to be constant. By using experimental column hold-up times to calculate the carrier gas velocity and taking into account the change in viscosity and gas compressibility with temperature, the total distance traveled by a solute can be calculated by summation of the distance traveled in the individual time segments. The solute retention time in the specified program is equivalent to the sum of the individual time segments corresponding to a migration distances equal to the column length. The above methods are flexible enough to allow simulation of retention for temperature programs containing different program rates and isothermal periods. Further equations allow prediction of peak widths so that simulated chromatograms allow resolution functions to be used to identify optimum separation conditions.

A significant advance in the simulation of temperature program separations was the demonstration that the linear-solvent-strength model developed for gradient elution liquid chromatography (section 4.4.6) could provide accurate predictions of temperature program retention times [242-244]. The measurement of retention times in two experimental linear temperature programmed separations over the same temperature range at different program rates provides the required experimental data. Ideally the program rates should differ by at least a factor of three. To simplify the calculations it is assumed that the column pressure drop and column hold-up time are constant and the column efficiency for all solutes is constant and independent of the separation conditions (isothermal or temperature program). At best crude approximations, but in reality the agreement between real and simulated chromatograms does not seem to have suffered too badly. The relationships used to estimate retention times for different program rates and isothermal periods are summarized in Table 2.14. The initial program conditions are used to calculate the program-steepness parameter, b , and the initial value of the retention factor for the temperature program, k_o , for each solute by numerical solution

of the retention time equation. The retention factor and the programming-steepness parameter combined with the initial experimental conditions allow the constants A' and S (related to the thermodynamic parameters of the system) to be estimated, from which separations at other conditions can be simulated. Data recorded for the initial temperature program separations can also be used to predict optimum isothermal separation conditions. Typical errors in simulated temperature program chromatograms are about $\pm 1-5\%$ for retention times, $\pm 5\%$ for bandwidths and $\pm 10\%$ for resolution [245-247].

2.4.4 Retention Index Systems and their Applications

Since the early days of gas chromatography an enormous effort has been devoted to standardizing methods used to determine retention data to make possible the wider use of collated published results for identification purposes. From the many proposals a general consensus has emerged in favor of the use of relative retention data expressed with respect to the retention of a standard substance. However, since gas chromatographic separations are performed over a wide range of temperatures with phases of different polarity, no single substance would fulfill the role of a universal standard. Also, in those cases where the retention of the sample and standard are grossly different accuracy would be impaired. This problem was finally solved by Kovats who proposed the retention index scale, in which the retention behavior of any substance was expressed with respect to the retention properties of a series of closely related standard substances [248-251]. The standard substances were the *n*-alkanes, each one of which was assigned an index value of 100 times its carbon number (100 for methane, 200 for ethane, etc.) at any temperature and on any phase. For members of a homologous series the logarithm of the adjusted retention time on a given column at a given temperature are linearly related to the carbon number if the first few members of the series are excluded. This provides the form of the relationship relating adjusted retention times to the fixed points of the retention index scale. The retention index of any substance can then be defined as equal to 100-times the carbon number of a hypothetical *n*-alkane which would have the same adjusted retention time as the substance of interest. The retention index is represented by the symbol I and is calculated under isothermal conditions using Eq. (2.5)

$$I = 100z + 100[\log t_R'(x) - \log t_R'(z)] / [\log t_R'(z+1) - \log t_R'(z)] \quad (2.5)$$

where t_R' is the adjusted retention time, z the carbon number of the *n*-alkane eluting immediately before the substance of interest denoted by x , and $z+1$ the carbon number of the *n*-alkane eluting immediately after substance x . Thus the retention index of a substance is expressed on a uniform scale with increased precision due to the use of two closely eluting and bracketing standards for the experimental measurement. For standardization of reporting it is recommended that the retention index be indicated by I with the stationary phase identified as a superscript, the sample as a subscript, followed

by the temperature of the measurement in parenthesis. In theory the retention index of a substance depends only on the temperature and the identity of the stationary phase and is independent of other instrumental and experimental parameters. In practice, this is an over simplification, as we shall see later.

Eq. (2.5) is the most common expressions for the retention index, although the same values are obtained by replacing the adjusted retention times by the specific retention volumes, gas-liquid partition coefficients, or the Gibbs free energies of solution when the retention mechanism is gas-liquid partitioning [249,252]. The assumption that the Gibbs free energy of solution for a methylene group is a constant for members of a homologous series is reasonable, if not exact, if the first few homologs are exempted [253,254]. Inaccuracies in the index values are likely to be greatest when the carbon number for the n-alkane standards is small, less than about five. Also, on polar phases the partial molar Gibbs free energy of solution for a methylene group is smaller than for a non-polar phase compressing the scale in the direction of increasing polarity. This results in lower relative retention of the n-alkane standards and a decrease in the relative measurement precision. A number of non-linear fitting procedures have been proposed to improve the accuracy of retention index measurements by accommodating the slight curvature in the retention plots against carbon number for the n-alkane standards [249,251,253-355]. A lack of consensus prevents any single method from being recommended.

The reliability of the retention index value is strongly dependent on the retention mechanism. Eq. (2.5) will be independent of the column characteristics only when retention occurs solely by gas-liquid partitioning. On polar phases the n-alkanes are often retained by a mixed retention mechanism with a significant contribution from interfacial adsorption (section 2.4.1). Under these circumstances the retention index values are unsuitable for qualitative identification and may well show variations between different laboratories running to hundreds of index units. The retention index concept is not restricted to the use of n-alkanes as index standards and any homologous series that shows a linear relationship between the logarithm of the adjusted retention time and carbon number can be used [256]. Standards of intermediate polarity such as 2-alkanones, alkyl ethers, alkyl halides, alkyl acetates and alkanolic acid methyl esters are less likely to be retained by interfacial adsorption on polar phases and will give more reliable index values. In other cases standards have been selected because they are normally present in the samples to be analyzed or are of a similar structure to the compounds to be determined and should have similar properties in the chromatographic system. For example, alkanolic acid methyl esters are used in lipid analysis; polychlorinated biphenyl congeners with different numbers of chlorine atoms for the identification of polychlorinated biphenyl isomers; and naphthalene, phenanthrene, chrysene and picene for polycyclic aromatic compounds [256-260]. A second reason for choosing index standards other than n-alkanes is to obtain compatibility with a selective detector that does not respond to the n-alkanes. Examples include n-alkyltrichloroacetates, n-bromoalkanes, and dichlorobenzyl alkyl ethers for use with the electron-capture detector; tri-n-alkylamines and 1-nitroalkanes with the thermionic ionization detector; di-

alkylsulfides with the flame photometric detector; and multifunctional standards for use with several detectors, such as alkyl bis(trifluoromethyl)phosphine sulfides [256]. Provided that a linear relationship between the new retention index standards and carbon number is obtained then it should be possible to develop a correlation equation to convert the measured retention index values into n-alkane retention index values, if desired.

When an isothermal index value is required at a temperature other than the temperature it was measured at it can be obtained by interpolation using several empirical relationships like the hyperbolic function

$$I(T) = A + B / (T + C) \quad (2.6)$$

where $I(T)$ is the retention index at any temperature $T(K)$ and A , B and C are experimentally derived constants [261,262]. The curve can nevertheless have a significant linear portion, particularly for substances of low polarity on non-polar stationary phases. For mixtures of a wide boiling point range the determination of retention indices under isothermal conditions would be time consuming and unnecessarily restrictive. Under temperature program conditions, there is an approximately linear relationship between the n-alkane elution temperature and their carbon number [249-251,263,264]:

$$I = 100z + 100[T_R(x) - T_R(z)] / [T_R(z + 1) - T_R(z)] \quad (2.7)$$

where T_R is the elution temperature (K) and the subscripts x , z , $z + 1$ are identified in Eq. (2.5). Since the elution temperature and retention time are usually highly correlated in linear temperature program separations the retention time can be substituted for the elution temperature in most cases. In practice, application of Eq. (2.7) requires that the initial column temperature is low compared to the elution temperature of the first standard and only a relatively limited range of carbon numbers are considered

The approximate nature of the relationship between carbon number and elution temperature or retention time has resulted in a large number of non-linear curve-fit methods for improving the accuracy of index measurements [265,266]. Curve fitting procedures are preferable when the range of carbon numbers for the standards is large or a linear equation results in a relatively poor fit to the experimental data. Temperature program retention indices are more sensitive to the chromatographic conditions than isothermal indices and are generally of lower precision. Temperature program indices are influenced by the characteristics (time and temperature) of any isothermal period before the start of the program, the temperature program rate (faster program rates lead to higher elution temperatures), and differences in column dimensions [267]. It is suggested for meaningful reporting of temperature program retention indices on open tubular columns that the stationary phase, film thickness, column length and internal diameter, initial temperature, program rate and column manufacturer are identified. Operation at constant pressure, constant flow or constant velocity has little influence on the retention index values.

A considerable effort has been devoted to predicting temperature program indices from the more readily available isothermal retention indices and their temperature dependence [235,240,248,251,263,264]. However, the unique dependence of the solute and n-alkane partition coefficients on temperature and the complex dependence of the column hold-up time on operating conditions have prevented any simple means of converting isothermal retention indices to program indices (and *vice versa*) from being developed. Several moderate size databases of temperature-program retention indices exist but are of limited use for identification purposes because of a lack of standardization [268,269]. An empirical approach to merging separate databases on similar stationary phases into a single database by making them statistically equivalent has been developed. The founding core was determined on an OV-1 open tubular column at a program rate of 8°C / min (Sadtlter database). Experimental conditions were then established for a representative and divers subset of compounds allowing other databases and the kernel to be merged into a new database, utilizing a plot of empirically determined retention index offsets for each data set. Individual search windows for the new indices were assigned from the 95 % confidence limits derived from the polynomial regression fit to the offsets. The new database has retention indices for over 3000 diverse structures. The use of retention index databases for compound identification is not that common [270]. For example, the percentage of correct identifications obtained by retention index matching of essential oil compositions on a single stationary phase was about 65 % increasing to 80 % if index values on two complementary stationary phases were used [267]. Typically, retention indices are used in combination with mass spectrometry to aid in the elimination of possible structures derived from searching mass spectral databases.

Sources of error in determining retention indices are due primarily to: poor control of fluctuation in temperature and carrier gas flow rates; non-ideal behavior of the carrier gas; uncertainty in the measurement of retention times and the column hold-up time; sample size effects; and variations caused by interfacial adsorption and impurities in the stationary phase [248-251,256,267]. Proper instrument design should minimize fluctuations in temperature and carrier gas flow rates. A significant systematic error can be caused by differences in the set point temperature of different instruments, which might, unknowingly, differ, by several degrees. For normal carrier gases and typical operating pressures non-ideal behavior should not be a significant source of error (section 1.4.1). Stationary phase purity speaks for itself, but columns can become contaminated in use, modifying their retention properties, and differences in stationary phase composition from different sources are possible, particularly since most common stationary phases are polymers with an inexact composition. If interfacial adsorption of solutes or standards is significant then wide variations in the retention index values can be expected. Retention indices in this case vary with the sample size, particularly the concentration ratio of the test substance to the n-alkanes, and with the phase loading or film thickness of open tubular columns. Rather than treat these observations as exceptional, as is the case frequently made in the literature, they are simply a consequence of the general retention model (section 2.4.1). For normal operating

conditions the reproducibility of retention indices between different laboratories is usually within a few index units for non-polar phases and within about 10-50 index units for polar phases; the precision for temperature programmed indices is generally lower. The reproducibility of retention indices within the same laboratory is generally better than a few index units.

2.4.4.1 *Quantitative structure – retention index relationships*

There has been a great deal of interest in the relationship between solute structure and retention for several reasons [248,249,251,271]. If predictive relationships could be developed then a method would exist to test the reliability of retention data, to predict the retention index of an analyte that was unavailable for study, or to predict the value of certain physical properties of an analyte that could be correlated through its retention index.

One such predictive relationship is the basis of the retention index system itself, that is that the members of a homologous series differ by 100 index units per methylene group (the first few members of the series excepted). Interpolation or precautionary extrapolation of the chromatographic data between identified members of a homologous series and suspect members establishes the probability of a substance in the chromatogram belonging to a particular homologous series. On low polarity stationary phases the retention increment due to the presence of a functional group relative to the parent hydrocarbon can be used to predict the retention index of structurally related compounds with reasonable accuracy [272,273]. If the retention index of a substance of the type alkyl chain-functional group is determined then subtraction of the value for the corresponding hydrocarbon having the same carbon number as the substance of interest gives the contribution for the functional group. The retention index for a compound on the same stationary phase can then be predicted by summation of the functional group increments to the base value predicted from its carbon number. For multifunctional compounds second order interactions lower the predictive accuracy. The method can be extended to polar phases by including a contribution from the retention index difference between the polar stationary phase and a nonpolar stationary phase.

More general approaches to retention index prediction are based on the generation of topological, geometric and electronic molecular descriptors, which are subsequently fit to the retention index using multiple linear regression or artificial neural networks [251,271-274]. For compounds of low polarity on nonpolar phases biparametric equations including solute boiling point combined with the van der Waals volume, molar volume, or the first-order molecular connectivity index have been quite successful. For polar solutes and/or polar phases additional terms must be included [275-280]. To account for contributions from polarizability and induction forces functions of the refractive index are generally used and for orientation forces functions of the dielectric constant or permanent dipole moment. Energy and charge parameters calculated by quantum chemical methods were found to result in improved correlations for some compounds. The above methods often provide reasonable results for compounds

belonging to a homologous series but variable accuracy for heterogeneous mixtures. The prediction of retention indices is generally not sufficiently accurate for substance identification at present.

Several methods are available to represent the structural formula of solutes by a topological model [271,276,281]. The structural formula of a compound may be viewed as a molecular graph, where the vertices represent atoms and the edges represent covalent bonds. More than twenty different topological indices have been described in the literature with the molecular connectivity indices being the most popular. Since topological indices basically reflect molecular shape and size (thus accounting for dispersion interactions) reasonable predictive agreement for retention indices of compounds of low polarity on nonpolar phases has been obtained. For polar phases, it seems that a combination of topological indices with interaction parameters, similar to those discussed above, are more rewarding. The general accuracy of predictions, however, is variable.

2.4.4.2 Rohrschneider's and McReynolds' phase constants

The system of stationary phase constants introduced by Rohrschneider [282,283] and later modified by McReynolds [284] was the first widely adopted approach for the systematic organization of stationary phases based on their selectivity for specific solute interactions. Virtually all-popular stationary phases have been characterized by this method and compilations of phase constants are readily available [28,30]. Subsequent studies have demonstrated that the method is unsuitable for ranking stationary phases by their selectivity for specific interactions [29,102,285-287]. The solvation parameter model is suggested for this purpose (section 2.3.5). A brief summary of the model is presented here because of its historical significance and the fact that it provides a useful approach for the prediction of isothermal retention indices.

The system of phase constants is formulated on the assumption that intermolecular interactions are additive and their individual contributions to retention can be evaluated from differences between the retention index values for a series of prototypical solutes measured on the stationary phase to be characterized and squalane at a fixed temperature of 120°C. The prototypical solutes, Table 2.15, were selected to express dominant intermolecular interactions, but in practice, only the first five solutes identified by the symbols X' through S' are widely used [29]. It was further assumed that for each type of intermolecular interaction, the interaction energy is proportional to a value a, b, c, d, or e, etc., characteristic of each prototypical solute, and to a value X', Y', Z', U', S', etc., characteristic of the capacity of the stationary phase to enter into specific intermolecular interactions. The retention index difference, ΔI , is thus compiled of product terms as follows:

$$\Delta I = aX' + bY' + cZ' + dU' + eS' \quad (2.8)$$

$$I_{PH}^P = I_{SQ}^P + \Delta I \quad (2.9)$$

Table 2.15

Prototypical solutes used by McReynolds (Rorschneider solutes in parentheses) to characterize stationary phase properties

R_2 = excess refraction, π_2^H = dipolarity/polarizability, $\Sigma\alpha_2^H$ hydrogen-bond acidity, $\Sigma\beta_2^H$ = hydrogen-bond basicity and $\log L^{16}$ partition coefficient on hexadecane at 25°C.

Symbol	Solute	Solute descriptors				
		R_2	π_2^H	$\Sigma\alpha_2^H$	$\Sigma\beta_2^H$	$\log L^{16}$
X'	Benzene	0.610	0.52	0	0.14	2.786
Y'	1-Butanol (Ethanol)	0.224	0.42	0.37	0.48	2.601
Z'	2-Pentanone (2-Butanone)	0.143	0.68	0	0.51	2.143
U'	1-Nitropropane (Nitromethane)	0.242	0.95	0	0.27	2.894
S'	Pyridine	0.631	0.84	0	0.52	3.022
H'	2-Methyl-2-pentanol	0.180	0.30	0.31	0.60	1.963
J'	Iodobutane	0.628	0.40	0	0.15	3.628
K'	2-Octyne	0.225	0.30	0	0.10	3.850
L'	Dioxane	0.329	0.75	0	0.64	2.892
M'	cis-Hydrindane	0.439	0.25	0	0	4.635

where I_{PH}^P is the retention index for a prototypical solute P on the stationary phase to be characterized, PH; I_{SQ}^P the retention index for the same solute on the non-polar reference phase squalane, SQ; and ΔI the retention index difference equivalent to the contribution from polar interactions. To evaluate the phase constants numerically, McReynolds assigned values of 1 to each of the test solutes in turn. For example, for benzene, $a = 1$, $b = 0$, $c = 0$, $d = 0$, $e = 0$; therefore, X' is obtained from Eq. (2.9) being equal to ΔI for benzene. By repeating this process for each prototypical solute in turn, all the stationary phase constants X' through S' can be determined. This requires the determination of the retention indices of the five test solutes on the phase to be characterized and on squalane under identical and defined experimental conditions. The larger the numerical value of the McReynolds' phase constant the stronger the interaction expressed by that constant.

McReynolds' approach is unreliable for both practical and theoretical reasons, briefly summarized as follows [29,102,285-287]:

- (i) Several of the prototypical solutes are too volatile to provide accurate retention values on a wide range of phases of different polarity. Some solutes elute at or close to the column hold-up time
- (ii) The method used to calculate retention indices ignores the contribution of interfacial adsorption as a retention mechanism (section 2.4.1). On polar phases the n-alkane retention index standards are retained predominantly by interfacial adsorption. The retention index values are irreproducible and can no longer be related to solvation properties of the prototypical solutes, which are retained predominantly by a partitioning mechanism.

(iii) The retention index differences used as phase constants are composite terms, the magnitude of which depends on the retention of both the index standards as well as the prototypical solutes. Using different retention index standards results in different ranking of the phases by selectivity. The magnitude of the phase constants is determined largely by the difference in retention of the n-alkanes on the compared phases, and to a lesser extent that of the prototypical solute. On many phases very good correlations exist between the magnitude of the phase constants and the Gibbs free energy of solution for a methylene group.

(iv) Prototypical solutes with a single dominant intermolecular interaction do not exist (see descriptors in Table 2.15). Each solute expresses several interactions simultaneously and the solvation process cannot be elaborated on in fundamental terms from the assumed properties for these solutes.

Rohrshneider's approach is able to predict retention index values for solute's with known solute constants (a, through e) [283,288]. These are determined from ΔI values for the solute on at least five phases of known phase constants and solving the series of linear equations. The retention index of the solute on any phase of known phase constants (X' through S') can then be calculated from Eq. (2.8). The theoretical defects of the method for assigning intermolecular interactions do not apply to the prediction of retention index values. A mean error of about 6 index units was indicated in some calculations. The retention or retention index values for thousands of compounds can be calculated from literature compilations of solute descriptors and the system constants summarized in Tables 2.6 and 2.8 using the solvation parameter model [103]. The field of structure-driven prediction of retention in gas chromatography is not well developed at present and new approaches will likely emerge in the future.

2.4.4.3 Column hold-up time

The column hold-up time (or volume) is an indispensable measurement for the calculation of thermodynamic properties of the separation system (e.g. t_R' , k , V_g , K_L) and for the calculation of retention indices. In addition, exact knowledge of the hold-up time at various temperatures is required for the prediction of retention during temperature program operation from isothermal measurements. A number of methods have been proposed for the determination of the hold-up time [289,290], which can be roughly categorized as: (i) measurement of the retention time of an unretained solute; (ii) deduced from regression calculations based on the retention of members of a homologous series; (iii) from the front of the solvent peak at high temperatures; and (iv) by calculation from Poiseuille's law behavior of the carrier gas. Convenience dictates that the method most commonly used is the determination of the time required for an infinitesimal amount of non-absorbed gas introduced simultaneously with the sample (or under conditions identical to those used to separate the sample) to pass through the chromatographic system. The selected solute must have no sorption interactions with the stationary phase and be easily determined by the detector used to record the chromatogram. Some examples are nitrogen, hydrogen, helium, argon, neon or air with a thermal conductivity or mass spectrometer detector; methane with

the flame ionization detector; methylene chloride with the electron-capture detector; acetonitrile with the thermionic ionization detector; and sulfur hexafluoride with the flame photometric detector. The column temperature and the stationary phase are important considerations in deciding whether a solute is retained or not. The inert gases or air are generally considered to be unretained in most systems except for some specific adsorption phases at low temperatures, such as molecular sieves and porous polymers. Methane is minimally retained on many phases but provides an estimate of the hold-up time suitable for most applications. At temperatures greater than 100°C methane sorption on moderately polar and polar phases and on thin film ($d_f < 0.3 \mu\text{m}$) non-polar phases in open tubular columns is virtually nonexistent [291]. In the temperature range 30 to 120°C the maximum deviation from the air peak for methane was $< 0.2 \%$ for open tubular columns and 4-5 % for packed columns [292]. When the hold-up time is measured directly it contains a contribution from extracolumn sources, that may be significant in some instrumental arrangements. When this is not the case, the simultaneous introduction of the sample at the inlet and outlet of the column with a valve arrangement allows the true column hold-up time to be determined from the difference in the residence time of the solutes between the two injection sites [293]. Bruno has described a modified autosampler vial containing a permeation tube for the release of methane into liquid samples for routine automated data collection [291].

Mathematical methods of determining the hold-up time are based on calculations from carrier gas properties [292,294] or the use of regression relationships employing retained compounds belonging to a homologous series [289,290,295-298]. Direct measurement from carrier gas properties requires an exact knowledge of the column length and radius, the column inlet and outlet pressure, and the carrier gas viscosity at the column temperature. Of these parameters, the column radius is difficult to measure with the required accuracy to calculate hold-up times, and this method is probably better employed for the accurate determination of the column radius from experimental hold-up time measurements. Regression methods based on the linearity of the semilog plot of retention time against carbon number are only approximate because these plots are rarely truly linear and the retention properties of the first few members of the homologous series are often anomalous (section 2.4.4). The accuracy is improved by using a non-linear regression method, such as that shown in Eq. (2.10)

$$t_R(z) = t_M + \exp(B + Cz^D) \quad (2.10)$$

where $t_R(z)$ is the retention time of an n -alkane containing z carbon atoms and B , C and D are regression constants. The value for the hold-up time is deduced from the retention time of four (not necessarily consecutive) n -alkanes plus methane by a regression procedure that obtains least squares estimates of the parameters by minimizing the residual sum of squares. Eq. (2.10) is purely empirical but seems to provide a better fit to experimental data than earlier equations and results in estimates of the hold-up time in good agreement with accurate experimental values.

2.5 PREPARATION AND EVALUATION OF OPEN TUBULAR COLUMNS

In the contemporary practice of gas chromatography open tubular columns are more important than packed columns. Among the different materials used to fabricate open tubular columns fused silica is by far the most important, although this was not always the case. The technology enjoyed today is the result of more than 40 years of continuous improvement [299]. Few chromatographers prepare their own columns anymore and declining numbers possess the knowledge and skill for this demanding task. Since the early 1990s nearly all iterative improvements in column technology have come from manufacturing improvements and are not well documented. Today we have better columns than ever before, but know less about them, and can only complain if radical changes are made to the production process that alters their separation properties or if useful products are taken out of production. This is not an entirely satisfactory situation but one that is unlikely to be reversed. We will thus be somewhat brief in our discussion of column preparation methods and suggest references [72,109,300-303] for a detailed discussion. A new approach using sol-gel technology is not as thoroughly investigated as these older methods but is attractive because of its simplicity [304]. The sol-gel column technology combines column coating, deactivation and immobilization into a single step procedure through chemical bonding of the stationary phase to an interfacial organic-inorganic polymer layer that evolves on top of the original capillary surface. The sol solution is prepared from an alkoxide-based precursor, a hydroxyl-terminated stationary phase, a surface derivatizing reagent and catalyst dissolved in a suitable solvent. The capillary is filled with the sol solution and allowed to stand for a controlled time, after which the sol is expelled from the column. The column is dried by gas purging and then thermally conditioned. A solvent rinse of the column prior to first use completes the preparation procedure.

2.5.1 Drawing Columns with Capillary Dimensions

Most early studies were conducted using stainless steel tubing and later nickel tubing of capillary dimensions. These materials had rough inner surfaces (leading to non-uniform stationary phase films), metal and oxide impurities at their surface which were a cause of adsorption, tailing, and/or decomposition of polar solutes and because their walls were thick, enhanced thermal inertia that prevented the use of fast temperature programming. At present, virtually all open tubular columns are prepared from fused silica and to a lesser extent borosilicate and soda-lime glasses [300-302,305]. Fused silica is prepared by oxidizing pure silicon tetrachloride in an oxygen electric plasma torch, and is essentially pure silica, containing less than 1.00 ppm of metal impurities. Because of its high melting point and toxicity special equipment and precautions are required for column fabrication. The surface of fused silica glass is relatively inert, containing primarily silanol and siloxane groups. The presence of silanol groups is responsible for the residual acidic character of the glass.

Glasses with lower working temperatures than silica are prepared by adding metal oxides to the silica during manufacture. Thousands of glasses with a variety of

compositions are known, but for chromatographic purposes, only soda-lime (soft) and borosilicate (hard, Pyrex, Duran) glasses are important. Typical values of the bulk compositions of soda-lime glass are SiO_2 67.7%, Na_2O 15.6%, CaO 5.7%, MgO 3.9%, Al_2O_3 2.8%, BaO 0.8%, and K_2O 0.6%, and for borosilicate glass SiO_2 81%, B_2O_3 13%, Na_2O 3.0%, Al_2O_3 2.0% and K_2O 1.0%. However, for chromatographic purposes the surface composition of the glass is more important than its bulk composition, and may differ substantially from the bulk composition. Soda-lime glasses are slightly alkaline, due to the high content of Na_2O , while the borosilicate glasses are somewhat acidic as a result of the presence of B_2O_3 . The adsorptive and catalytic activity of the column materials can be attributed to the silica surface (silanol and siloxane groups) and to the presence of metal impurities at the surface, which can function as Lewis acid sites.

Glass capillary columns with internal diameters of 0.05 to 1.0 mm and outer diameters of 0.3 to 1.5 mm are prepared from glass stock, typically 4.0 to 10.0 mm outer diameter and 2.0 to 6.0 mm internal diameter, using a glass drawing machine [301,302,306]. The glass tube is fed into a softening furnace that is electrically heated to a temperature of 650 to 850°C and drawn out at the lower end by passage through a pair of motor-driven rollers. Capillary tubes of different dimensions are drawn by maintaining a fixed differential between the feed rate of the stock tube to the furnace and the pull rate of the softened tube, at the exit of the furnace. The capillary tubing emerging from the softening oven is coiled by passage through a heated bent pipe maintained at 550 to 625°C. The actual temperatures used for the softening oven and bent pipe depend on the wall thickness and composition of the glass.

The polymer-clad, fused silica columns of the type introduced by Dandeneau and Zerenner in 1979 are drawn by techniques similar to those used for manufacturing optical fibers on equipment not found in analytical laboratories [303,307,308]. A simplified diagram of a drawing tower is shown in Figure 2.14. The drawn capillary tubing is inherently straight but sufficiently flexible to be coiled on a spool for collection purposes. The thin-walled tube must be protected immediately from moist air or dust particles that promote the growth of fissures or cracks, making the capillary weak and friable. This is done by coating the outside of the capillary tube as it emerges from the softening furnace with a protective film of polyimide or aluminum. Passage through an electrically heated drying oven completes the coating process. Fused silica columns are produced at draw rates of about 1 m/s.

During the drawing process the fused silica is subject to various temperature changes that cause hydration and dehydration, yielding a material with an uncontrolled silanol concentration. Residual silicon tetrachloride, trapped in the fused silica preform, and oxides of nitrogen, formed at the high temperatures required to melt the fused silica, can result in the production of acidic impurities (HCl and HNO_3) on hydrolysis. Conditioning and hydrothermal treatment are generally required to remove acid impurities and provide a uniform and defined silanol concentration for column preparation [309,310]. These procedures are usually performed in the laboratory and are essential to minimize batch-to-batch variation in the properties of the fused silica

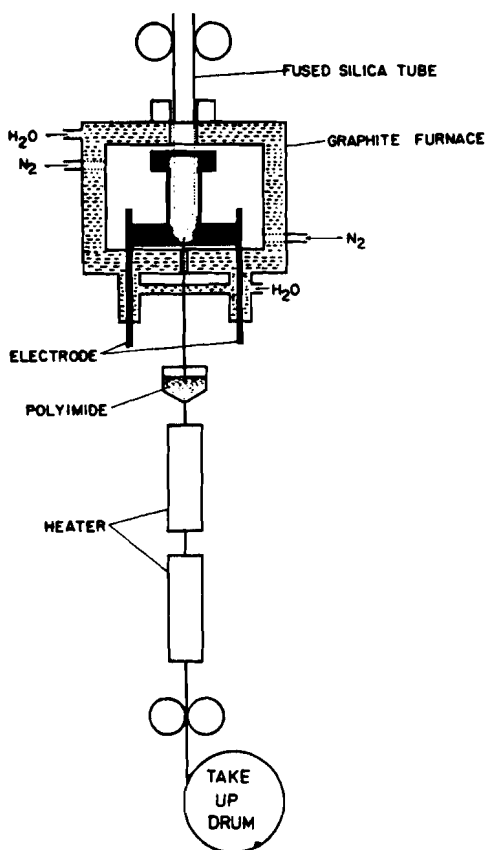


Figure 2.14. Schematic diagram of a drawing machine for producing polymer-clad, fused silica open tubular columns.

columns, which can lead to failure of optimized column deactivation and coating procedures found in the literature.

Fused silica capillaries are limited to some extent for high temperature applications by the thermal stability of the polyimide outer coating and for use in portable instruments and other applications where higher mechanical strength is desirable. Normal polyimide coatings are stable to about 350°C with newer materials having a reasonable service lifetime at 400°C. Aluminium has excellent high temperature stability but provides only modest protection of the fused silica [311]. Aluminium coatings act like a tube around a tube. Aluminium has a significantly different thermal expansion coefficient to fused silica and fails to form a hermetic wetted seal with the silica surface. Stainless steel capillary columns with an integral lining of silicon [312,313] or fused silica [314] provide the benefits of high temperature

stability and unsurpassed mechanical strength while approaching the low adsorptive and catalytic activity associated with fused silica. The silicon layer is prepared by the high temperature pyrolysis of silane in an inert atmosphere on an oxidized metal surface and the fused silica layer by decomposition of the inorganic polymer perhydropolysilazane in air. The fused silica layer is intimately bonded to the oxidized metal surface and is about 0.4 μm thick. Metal capillary columns with an interior coating of fused silica are commercially available but their method of production is not disclosed.

2.5.2 Film Formation on Glass Surfaces

To achieve high separation efficiency in any type of open tubular column it is essential that the stationary phase be deposited as a smooth, thin and homogeneous film that maintains its integrity without forming droplets when the column temperature is varied. To wet the glass surface the surface tension of the stationary phase should be less than the critical surface tension of the glass [315,316]. The stability of the film depends on the viscosity of the liquid and its resistance to disruption by forces tending to minimize the gas-liquid interface. Viscous films will provide greater stability whenever the time needed for the film to rearrange becomes long compared to the duration of the disturbance causing it to rearrange [310,317]. This is because the film is allowed insufficient time to break-up or form ripples under unstable conditions, and because the viscosity of the film acts as a dampening mechanism in opposition to the disturbance. To maintain temperature stability, phases showing little change in viscosity with temperature, such as the poly(siloxanes) (section 2.3.4), are preferred. All liquid films on curved surface are unstable in that the liquid film shows a natural tendency to decrease the interfacial energy at the gas-liquid interface leading to droplet formation, known as Rayleigh instability [318]. The logarithmic growth rate of Rayleigh instability is proportional to the surface tension of the liquid and to the third power of the film thickness, and inversely proportional to the viscosity of the liquid and to the fourth power of the column diameter. Therefore, wide bore and thin film columns show inherently greater stability and longevity than narrow bore and thick film columns.

The ability of a liquid to wet a solid surface is determined by the contact angle, that is, the angle formed between the tangent to the liquid drop and the solid surface as drawn through the liquid. For a liquid that completely wets the surface the contact angle is zero. When the cohesive forces of the liquid are greater than the adhesive forces between the liquid and the solid the contact angle increases. The contact angle, therefore, is an inverse measure of the wettability of the solid by the liquid. The surface tensions of typical stationary phases are in the range 30-50 dyne/cm. Clean fused silica and glass are high-energy surfaces and should be wet by most stationary phases. However, adsorption of moisture and other impurities generally lowers the critical surface tension to about 25-50 dyne/cm. Also, columns coated without deactivation of the surface often show undesirable activity towards sensitive analytes. Deactivation, then is a common column preparation procedure, performed to maintain or enhance the wettability of the surface.

Solutions to the above problem are required if efficient open tubular columns are to be prepared. The energy of the smooth glass surface can be increased by chemical

modification or roughening. Chemical modification is virtually the only approach used for fused silica columns while roughening and chemical modification is used individually or in combination with other glasses. Roughening, achieved by chemical treatment of the glass surface, enhances the wettability of the glass surface by increasing the surface area over which interfacial forces can act and dissipate the cohesive energy of the drop. Acid leaching or hydrothermal treatment restores the high energy of the glass surface, 40-50 dyne/cm, and provides a stable and uniform surface to achieve maximum deactivation. Deactivation is usually achieved by creating a bonded organic layer by silylation or by the thermal degradation of a polymeric substrate. Exhaustive silylation with a trialkylsilyl reagent forms highly deactivated surfaces of low energy, about 21-24 dyne/cm, which are suitable for coating with nonpolar stationary phases. For more polar phases silylation reagents containing functional groups similar to those in the stationary phase are used to maintain the desired degree of wettability. Formation of a non-extractable film of polar polymer, such as Carbowax 20M, by thermal treatment results in a high-energy surface, about 45 dyne/cm, which can be coated with many polar phases.

2.5.3 Surface Modification Reactions

Surface modification reactions are used to improve the wettability of glass surfaces by polar stationary phases and to improve the extent of deactivation by silylation [300-302]. The most important reactions are leaching with aqueous hydrochloric acid, etching by gaseous hydrogen chloride, formation of whiskers and solution deposition of a layer of solid particles. Because of the high purity and thinness of the column wall, leaching (hydrothermal treatment) is the only modification reaction generally used with fused silica columns.

Controlled acid leaching removes metallic cations from the surface of borosilicate and soda-lime glasses to form a silica-rich surface [300,319-321]. Leaching is typically performed by either static or dynamic treatment with 20 % (v/v) hydrochloric acid at 100-170°C. The new surface layer greatly minimizes the effects of glass variety on subsequent treatments and leads to a higher degree of reproducibility in column preparation. Lewis acid sites are removed, leaving highly adsorptive silanol groups; the latter are easily deactivated, for example, by silylation. Acid-leached borosilicate and soda-lime glasses are very inert after deactivation and can be coated with thin films of nonpolar phases. Since fused silica contains only traces of metal impurities deionization is not required but leaching is useful to optimize the concentration of surface silanol groups for uniform deactivation and possibly bonding in subsequent column preparation procedures [300,309]. The experimental conditions for the hydrothermal treatment of fused silica are often milder than that required for other glasses.

Etching, a different process to leaching, is performed almost exclusively with soda-lime glass capillaries and results in the formation of a regularly spaced array of sodium chloride crystals on the inner surface of the column. To etch soda-lime capillary columns the dry column is filled with hydrogen chloride gas, then sealed at both ends, and heated

at 300 to 400°C for different times (3 to 12 h) depending on the extent of the desired etch [322,323]. Borosilicate glasses contain a lower concentration of alkali metal ions and are little changed in appearance by hydrogen chloride treatment, unlike soda-lime glasses, which become opaque.

A variety of reagents and methods have been used for extending the surface area of borosilicate or soda-lime glasses by the preparation of whisker surfaces. A light surface etch can be obtained by flushing the capillary column with either an aqueous solution of potassium bifluoride [324] or a methanolic solution of ammonium bifluoride [325]. These surfaces are suitable for coating with non-polar and moderately polar stationary phases. Whisker columns of high density are prepared by the action of hydrogen fluoride gas at high temperatures on the glass surface. The preferred method is to generate hydrogen fluoride *in situ* by the thermal decomposition of 2-chloro-1,1,2-trifluoroethyl methyl ether [326-329] or ammonium bifluoride [330,331]. The surface area of whisker modified surfaces is about a thousand fold higher than the smooth glass surface and consists of a densely packed layer of filamentary projections whose size and length depend on the experimental conditions. The high surface area of whisker-wall columns enables any stationary phase to be coated efficiently without droplet formation. On the other hand, whisker surfaces are extremely active and very difficult to deactivate as well as being less efficient than WCOT columns.

Glass surfaces can also be roughened by deposition of a thin and homogeneous layer of particle material from solution. A dense layer of sodium chloride crystals can be deposited by dynamically coating columns with a stable sodium chloride suspension prepared by the addition of a saturated solution of sodium chloride in methanol to 1,1,1-trichloroethane or methylene chloride [332]. Coating with polar stationary phases produces efficient and thermally stable columns. Grob's barium carbonate procedure is probably the most widely evaluated of the particle deposition methods [300,333,334]. The general procedure consists of dynamically coating the glass surface with barium hydroxide solution using carbon dioxide gas to push the plug of hydroxide solution through the column and to generate the barium carbonate layer. From a saturated solution of barium hydroxide, a rather dense crystal layer is obtained that is suitable for coating with polar phases. From dilute solutions a very thin and smooth layer of barium carbonate is formed. In this case the barium carbonate probably forms a deactivation layer suitable for coating with nonpolar phases. General problems are the difficulty of completely deactivating the barium carbonate layer, poor thermal stability of some coated phases, and lower efficiency compared with other available methods.

2.5.4 Surface Deactivation Methods

Surface modification reactions used to improve the wettability of glasses by the stationary phase and to deionize some glasses, result in an increase in the activity of the glass surface. Without subsequent deactivation, columns coated with nonpolar and moderately polar stationary phases exhibit undesirable chromatographic properties, such as tailing, incomplete elution of polar solutes and in extreme cases, sample

decomposition. Although polar stationary phases may act as deactivating agents in their own right, column pretreatment is still advisable to ensure complete deactivation and to improve the thermal stability of the stationary phase film.

The effectiveness of a particular deactivating reagent depends on the properties of the glass, the identity of the stationary phase and the sample to be analyzed, as well as on the method of surface roughening. No universal deactivation method exists, but some techniques have emerged as more useful than others. The most widely used methods include high temperature silylation [335], reaction with poly(alkylhydrosiloxanes) [336,337] and reaction with the thermal degradation products of poly(siloxane) or Carbowax stationary phases [338-340]. High temperature silylation with disilazanes, disiloxanes, or cyclosiloxanes, thermal degradation of polysiloxanes, and the condensation of poly(alkylhydrosiloxanes) are now established as the most effective procedures for masking silanol groups on deionized glass and fused silica surfaces for subsequent coating with nonpolar and moderately polar poly(siloxane) stationary phases [300,302,335-337]. The R groups attached to silicon of the deactivating reagents, or a fraction of them, are generally selected to match the substituents attached to the siloxane backbone of the stationary phase [96,97,100,341-343]. Under typical conditions the capillary column is dynamically coated with the reagent or a solution of the reagent followed by removal of solvent, the column ends sealed, and the column heated to 250-300°C for the poly(alkylhydrosiloxanes) or 400-450°C for the other reagents. The column ends are then broken and the column purged with nitrogen at a high temperature to remove residual reagent and reaction products. At the high temperatures required for effective deactivation the polyimide external coating of the fused silica capillary columns must be protected with an inert atmosphere, since atmospheric oxidation decreases its flexibility and transparency.

Not all aspects of the high temperature silylation mechanism for glass surfaces are fully understood. There is probably a large commonalty in the way the various reagents react, although they were introduced and optimized separately before a more general picture emerged. Careful hydroxylation and dehydration of the glass surface is an essential prerequisite for optimizing the number of silanol groups and for establishing a fixed concentration of water which will be consumed during the silylation reaction. At moderate temperatures the steric bulk of the trimethylsilyl group prevents complete reaction of all silanol groups resulting in a surface which retains some residual activity that is not useful for preparing inert, nonpolar columns. Above about 400°C several alternative reactions to simple silylation are possible. These include formation of siloxane bonds by condensation of neighboring surface silanol groups; nucleophilic displacement of the organic group from the bonded reagent by a neighboring silanol group to form surface-bonded cyclic siloxanes; and reactions with water, forming short chain polymers bonded to the surface [335,337]. These reactions result in a decrease in the number of silanol groups on the deactivated surface and the formation of a protective umbrella-type film, diminishing access of the analyte to the remaining unreacted silanols. The thermal decomposition of poly(siloxane) phases occurs with the liberation of cyclic siloxanes and this explains the equivalence of results observed for

deactivation by thermal degradation of polysiloxanes and the direct application of cyclic siloxane reagents. Deactivation with poly(alkylhydrosiloxanes) occurs primarily by condensation with silanol groups on the fused silica surface with formation of surface-to-polymer bonds [3336,337,344]. In addition, physically adsorbed water hydrolyzes silane bonds to silanols, which further condense with silane bonds or other silanol groups to form a highly crosslinked resin film.

2.5.5 Coating Techniques

The object of all coating techniques is the distribution of the stationary phase as a thin, even film that completely covers the glass surface [300-302]. Dynamic coating and static coating methods, with several individual variations, are used for this purpose. For dynamic coating, a suitable coating reservoir is charged with a solution of the stationary phase, 5-15% w/v, which is forced by gas pressure into the capillary column to be coated. When about 25% of the column volume is filled, the column is withdrawn from the coating solution, and gas pressure is used to force the liquid plug through the column at a linear velocity of 1-2 cm/s. To maintain a constant coating velocity a buffer capillary column, which is about 25% of the length of the column being coated, is attached to the outlet end of the capillary column. When all the coating solution has left the capillary column it is disconnected from the buffer column and the gas pressure increased to evaporate the solvent. The thickness and uniformity of the stationary phase film depends on the concentration, surface tension and viscosity of the coating solution; the velocity and constancy of the velocity of the coating solution; temperature; and the rate of solvent evaporation. The dynamic coating procedure is comparatively fast but suffers from the general weakness that it is difficult to predict in advance the stationary phase film thickness.

The mercury plug dynamic coating method was introduced to improve the reproducibility of the dynamic coating method [345-347]. A short plug of mercury is interposed between the coating solution and the driving gas. The mercury plug wipes most of the coating solution off the surface of the column wall as it moves through the column, leaving a more even and thinner film behind. Concentrated coating solutions (10-50% v/v) are used in this procedure, producing films that are more resistant to drainage during the drying process.

The static coating method yields efficient columns of predictable film thickness with gum or solid stationary phases [300,348-350]. Phases of low viscosity, however, cannot be successfully coated by the static method due to their propensity to flow and accumulate in the lowest portions of the capillary column during the relatively long time required for solvent evaporation. For static coating the column is filled entirely with a dilute solution, 0.02-4.0% v/v, of the stationary phase in a volatile solvent, which is then evaporated by sealing one end of the column and attaching the other to a vacuum source. The solvent is evaporated under quiescent conditions, leaving behind a thin film of stationary phase of thickness given by $r_c C / 200$, where r_c is the capillary column radius, and C the concentration of the coating solution on a v/v basis.

The principal practical problem with the static coating method is the breakthrough of gas bubbles when the column is placed under vacuum. These bubbles generally originate at the solvent/seal interface or are trapped in microcavities in the glass surface. Breakthrough can eliminate all or part of the coating solution from the capillary column. The creation of a solvent/seal air-free interface is very important. Numerous methods have been described for this purpose and can be grouped into two categories. Mechanical methods that include clamping, crimping, freezing, or placing a stopper in a short length of Teflon or silicone tubing attached to the column end [348,351]. Chemical sealants such as waxes, Apiezon grease, epoxy resins, or water glass, etc., which are sucked into the end of the column and allowed to harden before evacuation is begun [300-302]. Air bubbles trapped in microcavities or scratches, etc., in the glass surface can be dissolved by allowing the column to stand several hours before evaporation is commenced or by pressurizing the filled column to several atmospheres for a short time. Rapid, even small, changes in temperature result in uneven film deposition and columns should be thermostated during the coating process by immersion in a large volume water or oil bath. Only volatile solvents, such as pentane, dichloromethane and Freons, provide reasonable column preparation times at ambient temperatures. For example, a 20 m x 0.3 mm I.D. capillary column filled with dichloromethane requires about 15 h for complete evacuation. Pentane requires only about half this time [352,353]. Static coating of narrow-bore capillary columns at temperatures above the solvent boiling point has been used successfully to minimize column preparation time [310,352].

Evaporation at atmospheric pressure, called the free release static coating method, has not been as widely used as the vacuum method [302,354]. The experimental arrangement is similar to that for the vacuum method except that no vacuum source is required and a dampening capillary column is normally attached to the open end of the column to increase the restriction to vapor transport and minimize the occurrence of bumping. Temperatures above the solvent boiling point can be used and coating is comparatively rapid, particularly for long and/or narrow bore columns, requiring very long coating times using the vacuum technique.

2.5.6 Evaluation of Column Quality

Whether using homemade or commercial columns, the assessment of column quality is of importance to all chromatographers. Periodic testing using a standardized quality test is the best method of monitoring changes in column properties. Column quality is a rather loosely defined concept that embodies such criteria as efficiency, inertness and the thermal stability of the stationary phase film. By inertness we signify the absence of adsorptive and catalytic interactions that can lead to incomplete elution, peak tailing and degradation for labile and polar analytes. All these properties depend upon the method and materials chosen for column preparation.

The efficiency of an open tubular column can be measured in several ways, such as by the plate number (N), the effective plate number (N_{eff}), the plate height (H) or the

separation number (section 1.5). All these methods measure system efficiency, and if the values obtained are to be meaningful, sources of extracolumn band broadening have to be negligible. The plate number or effective plate number, usually normalized per meter of column length, is easily measured from an isothermal chromatogram for inert substances such as hydrocarbons, ketones or esters. The values obtained, of course, will vary with retention, column dimensions, carrier gas type and velocity, and stationary phase film thickness. Values measured for weakly retained solutes (low k) should not be used for column evaluation. For a particular carrier gas, the efficiency will maximize at an optimum velocity. Only at the optimum velocity are N , N_{eff} , and H independent of the column length. The column efficiency depends on the column diameter, see Table 2.2, where typical values of N and H for different column types can be found. Values for N and H are reasonably independent of temperature but may vary with the substance used for their determination, particularly if the test substance and stationary phase is not compatible.

2.5.6.1 Activity tests for uncoated columns

Activity tests for uncoated columns are important for studying the effectiveness of surface preparation and column deactivation procedures [100,355-358]. Even when using a well-tried method something may go wrong or the deactivation may be incomplete, and an intermediate test prior to coating is useful for recognizing problems of this kind. Test methods are based on the principle that if a symmetrical solute band passes through an inert and uncoated capillary column it should emerge from that column unchanged except for symmetrical band broadening, and its retention time in that column should be equivalent to the column hold-up time. To perform the test a short, coated capillary column of high quality is connected to the injector at one end and a short length of the capillary column to be tested at the other. The free end of the test capillary is connected to the detector. The test substances, after separation by the coated column, enter the test capillary as perfectly shaped elution bands. The procedure generates nearly Gaussian peaks, from which symmetry distortions may be mathematically evaluated using symmetry factors, peak area/peak height ratios, etc. A practical example is shown in Figure 2.15 for the separation of a test mixture of basic compounds on a deactivated fused silica capillary column compared to a piece of column prior to deactivation [355]. Almost any of the popular polarity test mixtures can be used to estimate the nature and extent of the activity of the test capillary column. The Grob test mixture (section 2.5.6.2), the mixture of basic compounds identified in the legend to Figure 2.15, and a mixture of polar and acidic compounds containing *n*-decane, 1-octanol, 2,6-dimethylphenol, 1,6-hexanediol, octanoic acid, *n*-dodecane, 2,4,5-trichlorophenol, 4-nitrophenol and *n*-heptadecane, or selected components from the above mixtures, are widely used.

2.5.6.2 Standardized quality test for coated columns

The comprehensive column test procedure devised by Grob is the most widely used standardized test for the comparison of quality for coated columns [300,359-361]. The

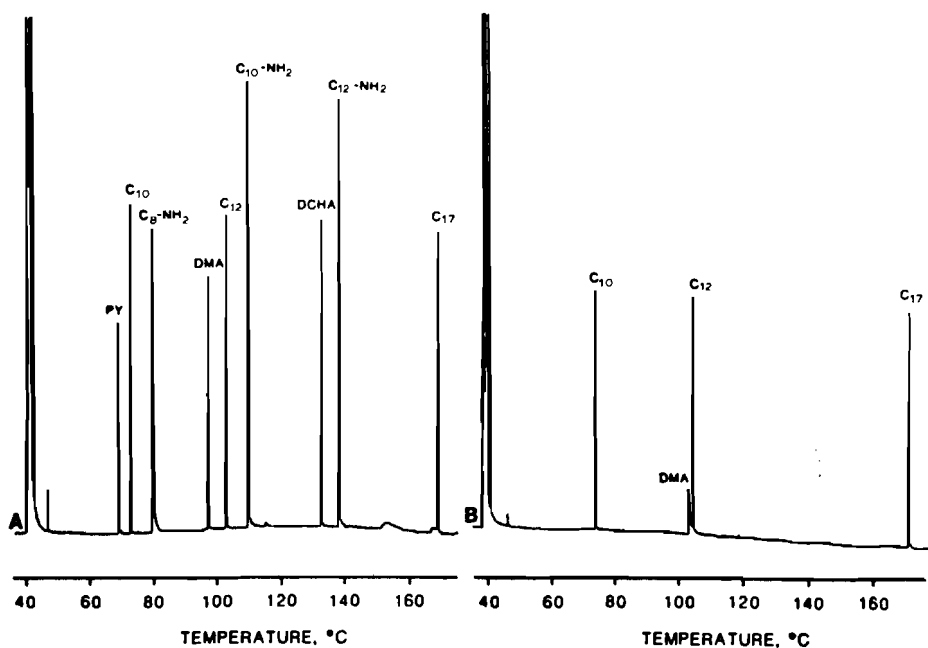


Figure 2.15. Activity test for an uncoated fused silica capillary column after (A) deactivation with poly(phenylmethylhydrosiloxane) and (B) before deactivation. Precolumn: 15 m x 0.20 mm I.D. coated with SE-54. Test columns 10 m x 0.20 mm I.D. The column tandem was programmed from 40 to 180°C at 4°C/min after a 1 min isothermal hold with a hydrogen carrier gas velocity of 50 cm/s. The test mixture contained C₁₀ = n-decane, C₈NH₂ = 1-aminooctane, PY = 3,5-dimethylpyrimidine, C₁₂ = n-dodecane, C₁₀NH₂ = 1-aminodecane, DMA = 2,6-dimethylaniline, DCHA = N,N-dicyclohexylamine, C₁₂NH₂ = 1-aminododecane, and C₁₇ = n-heptadecane. (From ref. [355]. ©Wiley-VCH).

Grob test provides quantitative information about four important aspects of column quality: separation efficiency, adsorptive activity, acidity/basicity and the stationary phase film thickness. The experimental conditions are optimized for columns of low polarity with a medium range of film thickness (0.08-0.4 μm) and column internal diameters of 0.25-0.35 mm. The composition of the Grob test mixture and experimental conditions for the test is summarized in Table 2.16. The individual standard solutions are stable almost indefinitely and the test mixture for several months when stored in a refrigerator. Reaction between nonanal and 2,6-dimethylaniline to form a Schiff base derivative occurs on prolonged storage resulting in reduced peak areas for these two compounds [362]. The test mixture is injected to allow ca. 2 ng of each substance to enter the column (e.g. 1 μl with a split ratio of 1:20 to 1:50).

The test is performed under optimized conditions of carrier gas flow and temperature program rates, which are adjusted for column length and carrier gas viscosity, Table 2.16. To obtain a correct value for the gas hold-up time for thick-film columns ($d_f > 0.7 \mu\text{m}$) it should be measured at 100°C (methane is considerably retained at

Table 2.16

Test mixture composition and optimum experimental conditions for the Grob test.

Test compounds dissolved in 20 ml of hexane except for 2,3-butanediol, which is dissolved in chloroform. Working solution is prepared by mixing 1.0 ml of each standard solution and diluting 1.0 ml of this solution to 20 ml in hexane. To reduce the likelihood of peak overlap on non-polar stationary phases n-dodecane is used instead of n-undecane.

Composition of concentrated test mixture

Substance	Abbreviation	Amount (mg)	Substance	Abbreviation	Amount (mg)
Methyl decanoate	E ₁₀	242	1-Octanol	ol	222
Methyl undecanoate	E ₁₁	236	Nonanal	al	250
Methyl dodecanoate	E ₁₂	230	2,3-Butanediol	D	380
n-Decane	10	172	2,6-Dimethylaniline	A	205
n-Undecane	11	174	2,6-Dimethylphenol	P	194
n-Dodecane	12	176	Dicyclohexylamine	am	204
			2-Ethylhexanoic Acid	S	242

Optimized experimental conditions

Carrier gas measurements at or close to room temperature. Initial temperature 40°C for program.

Column length (m)	Hydrogen		Helium	
	Methane elution (s)	Temperature program (°C/min)	Methane elution (s)	Temperature program (°C/min)
10	20	5.0	35	2.5
15	30	3.3	53	1.65
20	40	2.5	70	1.25
30	60	1.67	105	0.84
40	80	1.25	140	0.63
50	100	1.0	175	0.5

25°C). Since the viscosity of the carrier gas is greater at this temperature, the hold-up time should be corrected by adding 10% of the measured time for hydrogen and 15% for helium. Some instruments do not allow the temperature program rate to be changed continuously to meet all conditions given in Table 2.16. In this case it is necessary to correct the speed of the run to an available temperature program rate by selecting a gas hold-up time corresponding to this program rate. For thin film columns the sample is usually injected at or close to room temperature and the column temperature raised to 40°C and the appropriate optimized temperature program started.

The average separation number for the alkanolic acid methyl esters determines column efficiency. For thin films and moderately polar stationary phases the adsorptive and acid/base characteristics of a column can be quantified as the percentage of the peak height expected for complete and undisturbed elution. The two alkanes and three alkanolic acid methyl esters (non-adsorbing peaks) are connected at their apexes to provide the 100% line [333]. The column activity is then quantified by expressing the height of the remaining peaks as a percentage of the distance between the baseline and the 100% line. It accounts for all types of peak distortion that occur in practice: peak broadening, peak tailing, irreversible adsorption and degradation. On polar phases this is generally not applicable. The two alcohols are used to measure adsorption by

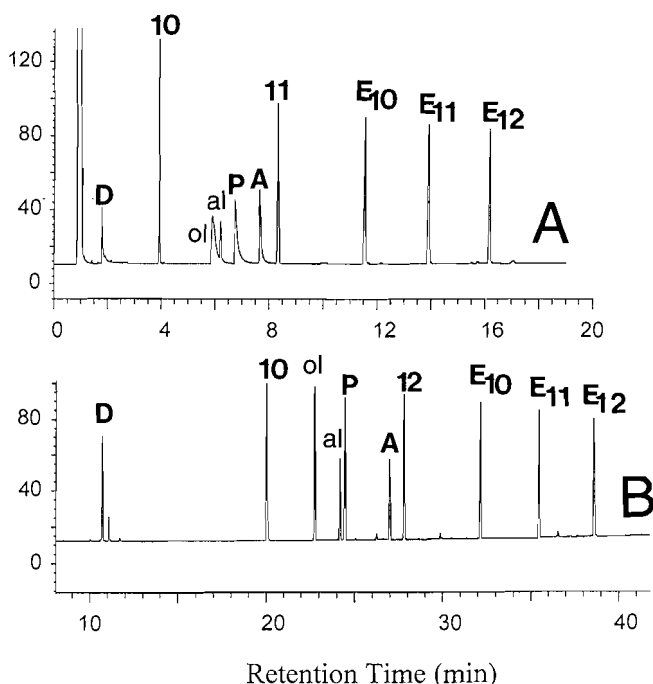


Figure 2.16. Example of column deterioration due to aging. The 0.25 mm I. D. fused silica columns are 15 m (A) and 30 m (B) coated with 1.0 μm DB-5 stationary phase. Column activity is determined using the Grob test method (Table 2.16). Column (A) is an old column exhibiting strong activity. Column (B) is a new column. The reduced peak heights of nonanal and 2,6-dimethylaniline on both columns also indicate change in relative composition of the test mixture due to storage time.

hydrogen bonding. Acid/base interactions are assessed from the adsorptive behavior of 2,6-dimethylaniline, 2,6-dimethylphenol, dicyclohexylamine, and 2-ethylhexanoic acid. 2-Ethylhexanoic acid and dicyclohexylamine provide a more stringent test of acid/base behavior than the phenol and aniline. On non-polar phases, even less than 1.0 ng of 2-ethylhexanoic acid may cause column overloading, resulting in a distorted (leading) peak, which should not be mistaken for adsorptive activity. The film thickness is calculated from the elution temperature of methyl dodecanoate. However, quantitation of film thickness requires a calibration of elution temperature against stationary phase film thickness for all stationary phases of interest. The stationary phase film thickness can then be obtained from the elution temperature of methyl dodecanoate [359].

Good columns eventually deteriorate over time due to modification of the stationary phase film or glass surface induced by moisture or matrix components introduced with the samples or by the carrier gas, thermal stress, attack by oxygen and aging. Column efficiency for inert compounds may remain acceptable but increasing adsorptive and catalytic activity eventually precludes the separation of polar compounds. Figure 2.16 shows an example of the influence of column deterioration on the separation of several

components of the Grob test mixture. The reduced peak height and tailing of the alcohols, 2,6-dimethylphenol and 2,6-dimethylaniline are typical characteristics of an active column.

The Grob test cannot be used to evaluate columns coated with high melting point stationary phases or base-deactivated stationary phases (due to decomposition of the alkanolic methyl esters and abstraction of acidic components from the test mixture) [363]. The elution order of the test compounds is not the same on all stationary phases and the occurrence of peak co-elution cannot be eliminated entirely. In the case of peak co-elution, the test mixture should be divided into groups of separated components and each group injected independently. Finally, the test is biased towards the measurement of adsorptive as opposed to catalytic activity [364,365]. Catalytic activity causes time-dependent, concentration-independent losses of sample components. With increasing column temperature, adsorption decreases whilst catalytic/thermal decomposition increases. Catalytic activity is often assessed using the Donike test employing a mixture of homologous alkanes and alkanolic acid trimethylsilyl esters [366]. The alkanes are inert compounds while the trimethylsilyl esters are very sensitive to catalytic degradation. A change in the relative ratio of the two homologous series provides an indication of the catalytic activity of the chromatographic system. The relative decomposition of triacylglycerols has been recommended for evaluating the catalytic activity of columns in high temperature gas chromatography (300-420°C) [367]

2.5.6.3 Column thermal stability

The bleed products from poly(siloxane) phases consist primarily of low molecular weight cyclic siloxane impurities and thermally induced cyclic siloxanes (section 2.3.4). The amount of volatile decomposition products formed in the column per unit time depends on the type and amount of stationary phase, temperature, carrier gas flow rate and surface properties of the column wall [301,367-369]. Standardization of the measurement of column bleed from different columns presents a number of problems. Injecting a standard compound as part of the test can eliminate the influence of detector sensitivity on the results obtained. The amount of stationary phase bleed is represented by a rectangle whose area is defined by the change in response between two set temperatures, one of which is selected to represent conditions of low column bleed, and the section of the abscissa that corresponds to unit time, Figure 2.17. By comparing the area of the rectangle with the area of a known amount of a test compound, usually an n-alkane, the loss of stationary phase can be expressed in units of mass per unit time (e.g. μg of n-alkane per minute). As a necessary working hypothesis it is assumed that the response factors of the column bleed products are identical to those of the standard compound. This method is suitable for studying the influence of the column preparation procedure on the stability of the stationary phase film. In general, soda-lime glass capillary columns exhibit greater column bleed than borosilicate glasses unless the concentration of alkali metal ions is reduced by acid leaching. Fused silica columns show very low levels of thermally induced catalytic phase decomposition.

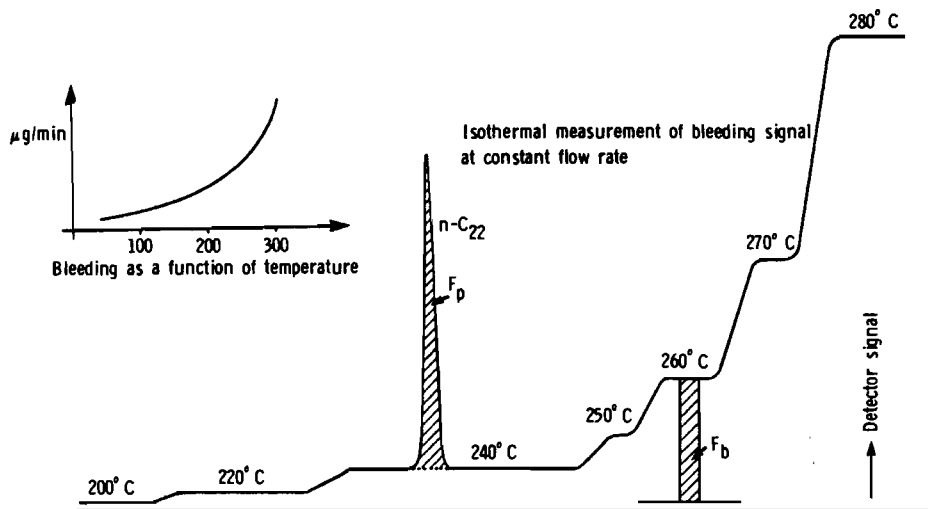


Figure 2.17. Standardized column bleed test. (From ref. [369]. ©Elsevier)

2.6 PREPARATION AND EVALUATION OF PACKED COLUMNS

Packed columns coated with a wide variety of stationary phases are commercially available. Unlike open tubular columns they are easily prepared in the laboratory and many chromatographers prefer to prepare their own columns. Almost any liquid not restricted by its vapor pressure can be studied extending the scope of packed columns to stationary phases unavailable on open tubular columns as well as allowing gas chromatography to be used to obtain physicochemical properties of a wide range of materials evaluated as stationary phases.

2.6.1 Supports

The ideal support would behave like a sponge holding the liquid phase as a thin film without playing any further role in the separation process. To eliminate solute interactions with the support its surface activity should be low, yet contrarily, the surface must have sufficient energy to hold the stationary phase in place and to cause it to wet the surface in the form of a thin film. The ideal support would also have a large surface area to weight ratio to allow for the use of high phase loadings and a regular shape, with a narrow range of cross-sectional diameters, so that columns of high efficiency can be prepared. In addition, it should be a good conductor of heat and mechanically and thermally stable to avoid changes in properties on handling and while in use. No such ideal support exists and a compromise between those properties, which are desirable, and those that can be obtained practically must be reached. By far the most important support materials are the diatomaceous earths [370-373].

Table 2.17

Characteristic properties of Chromosorb supports

Property	P	W	G	A	750
Color	pink	white	oyster	pink	white
Apparent pH	6.5	8.5	8.5	7.1	8.0
Free fall density (g/ml)	0.38	0.18	0.47	0.40	0.37
Packed density (g/ml)	0.47	0.24	0.58	0.48	0.36
Surface area (m ² /g)	4.0	1.0	0.5	2.7	0.5-1.0
Maximum liquid loading (%)	30	15	5	25	12

Diatomite (diatomaceous earth, Kieselguhr) is composed of the skeletons of diatoms, single-celled alga, which have accumulated in large beds in various parts of the world. The skeletal material is essentially amorphous silica with small amounts of alumina and metallic oxide impurities. In their native form the diatomite skeletons are too small and fragile to be used as chromatographic supports. Calcining at temperatures in excess of 900°C is used to agglomerate and strengthen the natural material. The presence of complex iron oxides gives this material its characteristic pink color. Close inspection of the processed material reveals a compact mass of broken diatomite fragments with a portion of the secondary pore structure still intact. Calcining diatomite in the presence of a small amount of sodium carbonate results in the formation of a white material in which metal impurities are converted to colorless sodium silicates. Close inspection reveals a mass of large diatomite fragments held together by sodium silicate glass with most of the secondary pore structure destroyed. Thus the white material has a smaller surface area when compared to the pink and the addition of sodium carbonate imparts a slightly basic character to the material compared to the natural acidic character of the pink material. The bulk chemical composition of the two types of diatomaceous earth are quite similar (SiO₂ 88.9-90.6%, Al₂O₃ 4.0-4.4%, Fe₂O₃ 1.6%, CaO 0.6%, MgO 0.6%, and Na₂O + K₂O 1.0-3.6%) [370].

The physical properties of the popular Chromosorb supports are summarized in Table 2.17. The pink supports are relatively hard, have a high packing density, large surface area and high loading capacity. They are used in both analytical and preparative gas chromatography. The white supports are more friable, less dense, have a smaller surface area and loading capacity, and are used in analytical gas chromatography. Chromosorb G is a specially prepared white support that is harder, more robust, and inert than Chromosorb W. It has a low loading capacity but higher density than the regular white supports. A given column volume contains approximately 2.5 times the amount of Chromosorb G, and therefore liquid phase, as Chromosorb W of the same nominal phase loading. Chromosorb A, a pink support developed specifically for preparative gas chromatography, possesses the mechanical strength and high stationary phase capacity of pink supports with a reduced surface activity approaching that of white supports. Chromosorb 750 is a white support, which is chemically treated after processing and is the most inert of the Chromosorb series.

Gas chromatographs are designed to operate at column inlet pressures of a few atmospheres. The column pressure drop is inversely proportional to the square of the

Table 2.18
Particle size ranges for diatomaceous supports

Mesh range	Top screen opening (μm)	Bottom screen opening (μm)	Spread (μm)	Range ratio
10-20	2000	841	1159	2.38
10-30	2000	595	1405	3.36
20-30	841	595	246	1.41
30-40	595	420	175	1.41
35-80	500	177	323	2.82
45-60	354	250	104	1.41
60-70	250	210	40	1.19
60-80	250	177	73	1.41
60-100	250	149	101	1.68
70-80	210	177	33	1.19
80-100	177	149	28	1.19
100-120	149	125	24	1.19
100-140	149	105	44	1.42
120-140	125	105	20	1.19
140-170	105	88	17	1.19
170-200	88	74	14	1.19
200-230	74	63	11	1.19
230-270	63	53	10	1.19
270-325	53	44	9	1.20
325-400	44	37	7	1.19

particle diameter, whereas the column efficiency is directly proportional to the particle diameter. For analytical purposes supports with a mesh range of 80-100 or 100-120 are a reasonable compromise. The highest efficiencies are obtained with particles of a narrow mesh range. Traditionally, particle size range is quoted in terms of mesh and the designation 100-120 mesh means that the particles passed through a sieve of 100 mesh but not a sieve of 120 mesh. The mesh range in units of micrometers is given in Table 2.18.

Severe tailing of polar compounds, decomposition, structural rearrangements and even complete sample adsorption demonstrated that the crude diatomaceous earth supports were not sufficiently inert for use in biomedical and environmental applications. These undesirable interactions were associated with the presence of metallic impurities and silanol groups at the surface of the diatomaceous supports. Acid and/or base washing to remove metallic impurities and silanization of surface silanol groups are presently the most widely used methods of support deactivation. Surface metallic impurities, principally iron, are removed by soaking or refluxing with 6N hydrochloric acid [374]. Surface silanol groups are converted to silyl ethers by reaction with a silanizing reagent such as dimethyldichlorosilane (DMCS), hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS), or a combination of these reagents. Silanization changes the character of the support from hydrophilic to hydrophobic. Consequently, silanized supports are unsuitable for preparing packings with

polar stationary phases. The treatment undergone by the diatomaceous support is normally stated on the manufacturer's label, for example, Chromosorb W-AW-DMCS indicates that the white support was acid washed and silanized with dimethyldichlorosilane.

The most extensive chemical treatment and silanization of diatomaceous supports cannot remove completely those active centers which cause tailing of strongly acidic or basic compounds. The addition of small quantities of "tailing reducers" is necessary for the successful analysis of these compounds. Tailing reducers are coated onto the support in a similar manner to the stationary phase or with the stationary phase, and to be effective, they must be stronger acids or bases than the compounds to be separated. For amines, the tailing reducer could be a few percent of potassium hydroxide or poly(ethyleneimine) [371,375,376]. For acidic compounds phosphoric acid, FFAP (section 2.3.2), or trimer acid are suitable [371]. These active substances will also act as subtractive agents and thus an acidic tailing reducer will remove basic substances from the chromatogram and vice versa. The phase itself must also be compatible with the reagents. Potassium hydroxide and phosphoric acid, for example, catalyze the depolymerization of poly(ester) and poly(siloxane) phases.

Other support materials of minor importance include various fluorocarbon powders (Teflon-6, Kel-F, Fluoropak-80, etc.), glass beads, carbon and dendrite salt [370-373]. The fluorocarbon powders have inert and low energy surfaces unsuitable for coating with polar phases. They are used primarily as supports for fluorocarbon liquids (section 2.3.1) to separate reactive compounds that would destroy or be destroyed by other materials. Teflon supports develop electrostatic charges and are difficult to handle for coating and packing above 19°C (solid transition point). Glass beads are available as spheroids in narrow mesh ranges suitable for preparing efficient columns. Their low surface energies limit phase loadings to less than 0.5% w/w. At higher loadings the column efficiency is reduced due to the formation of pools of stationary phase at the contact points of the beads. The glass surface is active and tailing of polar compounds is observed [377]. Silanizing the beads reduces tailing caused by silanol groups but also diminishes the loading capacity. Glass beads are used mainly in theoretical studies because of their controlled shape and size and for very rapid analyses where their low loading capacity is not a disadvantage.

2.6.2 Coating and Packing Techniques

Supports are usually coated with stationary phase using one of several evaporation methods. In outline, the stationary phase in a suitable solvent is mixed with the support, the solvent removed, and the dried packing added to the empty column with the aid of pressure or suction [67]. The coating solvent should be a good solvent for the phase and of sufficient volatility for easy evaporation. High purity solvents without stabilizers should be used so as not to contaminate the packings. Recommended solvents for coating are usually indicated by the manufacturer of the stationary phase, and although other solvents can be used, a poor choice of solvent can lead to uneven coating and

packings of below average efficiency. By convention, column packings are prepared on a weight per weight basis and quoted as percent liquid phase. Typical phase loadings range from 0.5 to 30% w/w depending upon the type of separation. The maximum useful phase loadings for various supports are given in Table 2.17.

The most common coating procedure is the rotary evaporator technique. The support is coated by adding it slowly to a fluted flask (powder drying flask) containing the stationary phase in sufficient solvent to completely wet the support and form a layer above it. The support is conveniently added to the hand swirled solution through a fluted paper with a small hole at its center. The solvent is then removed in the usual way on the rotary evaporator being careful to avoid rapid rotation of the flask or violent bumping of the damp solid. Alternative coating techniques include: filtering a slurry of the stationary phase and solid support through a Buchner funnel; packing the support into a column containing a glass frit and passing a solution of the stationary phase through the bed of support aided by overpressure or suction; and the pan-dry method, in which the slurry is gently stirred in a large flat glass dish under an infrared heating lamp or stream of nitrogen to aid evaporation. In all cases it is important to treat the packings gently to avoid the formation of fines.

After coating the damp support may be air dried, oven dried or dried in a fluidized bed dryer. Fluidized bed drying is faster and may lead to more efficient and permeable column packings, since fines developed during the coating procedure are carried away by the gas passing through the packing [67,378]. The dried packing may be mechanically sieved to remove fines. Dry sieving is easy to perform but may also cause extensive attrition of the fragile diatomaceous supports. Mechanical sieving prior to coating (unopened bottles of support may contain a large volume of fines) and fluidized bed drying after coating are the preferred methods of minimizing the deleterious effects of fines on column performance. For general purposes it is assumed that the ratio of stationary phase to support does not change during the coating process. The amount of stationary phase coated on the support, however, is frequently less than the nominal amount weighed out due to losses of phase to the walls of the coating vessel and from uneven coating. The latter tends to result in heavily loaded particles adhering to the walls of the coating flask that remain behind after the coated support is removed. For physicochemical property measurements the exact weight of liquid phase in the column must be known. This is usually determined by either combustion, evaporation or Soxhlet extraction of a sample of the column packing [379,380]. For Soxhlet extraction, ca. 0.5-1.0 g of accurately weighed packing in a fine porosity glass sintered crucible with a small piece of filter paper on top, is placed in a standard Soxhlet apparatus. Glass beads are used to raise the level of the crucible above that of the siphon arm. The packing is then extracted at rate of about 10-15 cycles per hour with, generally, the same solvent used for coating. Quantitative extraction of the stationary phase usually requires about 4 hours but overnight extraction is generally used to ensure completion. After extraction the packing is oven dried to constant weight.

Stainless steel, nickel, or glass tubes bent into various shapes to fit the dimensions of the column oven provide the container for column packings [381]. Stainless steel

is adequate for nonpolar compounds but its catalytic activity precludes the analysis of labile substances. Nickel (after acid passivation) and glass are the most inert column materials, and are preferred for the analysis of labile compounds. Teflon or plastic tubing is also used occasionally, often in the separation of chemically reactive substances for which Teflon supports are also required. Low upper temperature limits and permeability to some solutes restricts their general use.

Columns 0.5-3.0 m long with 2-4 mm internal diameters can be conveniently packed by the tap-and-fill method aided by suction. One end of the column is terminated with a short glass wool plug and attached via a hose to a filter flask and water pump aspirator [67]. A small filter funnel is attached to the other end and the packing material is added in small aliquots. The column bed is consolidated as it forms by gentle tapping of the column sides with a rod or with the aid of an electric vibrator. Excessive vibration is detrimental to the preparation of an efficient column and should be avoided. When sufficient packing has been added the funnel is removed and a glass wool plug is inserted. The length of the plug is usually dictated by the design of the injector. Columns longer than 3.0 m are difficult to pack and require a combination of vacuum suction at the free end and pressure applied to the packing reservoir at the other end [382].

The packed column requires a temperature conditioning period before use. The column is installed in the oven with the detector end disconnected. The column temperature is then raised to a value about 20°C higher than its intended operating temperature while a low flow of carrier gas is passed through it. In all cases the temperature used for conditioning should not exceed the maximum operating temperature for the phase. The conditioning period is complete when a stable detector baseline can be obtained. Columns are often conditioned overnight prior to initial use.

2.6.3 Evaluation of Column Quality

After conditioning the column a few preliminary tests are performed to ensure that the performance of the column is adequate. A measure of column efficiency is made with a test sample, for example, a mixture of n-alkanes, Figure 2.18. An average plate count of about 2000 per meter is acceptable for a 10% (w/w) loaded column on 80-100 mesh support. Higher values are expected for columns with lower loadings and with finer mesh supports. Columns with a plate count less than 1500 per meter are of doubtful quality. An unusually high column inlet pressure indicates problems associated with attrition of the column packing.

After testing the efficiency of the column, some test of column activity and resolving power may be made. A polarity test mixture reflecting the polar functional groups of the samples to be separated is selected to assess column activity. Excessive tailing or adsorption of the test compounds indicates residual column activity. Interactions with support silanol groups can often be reduced by on-column silanization using one of the many available column-conditioning reagents. To test the resolving power of the column a mixture that reflects the intended use of the column should be selected.

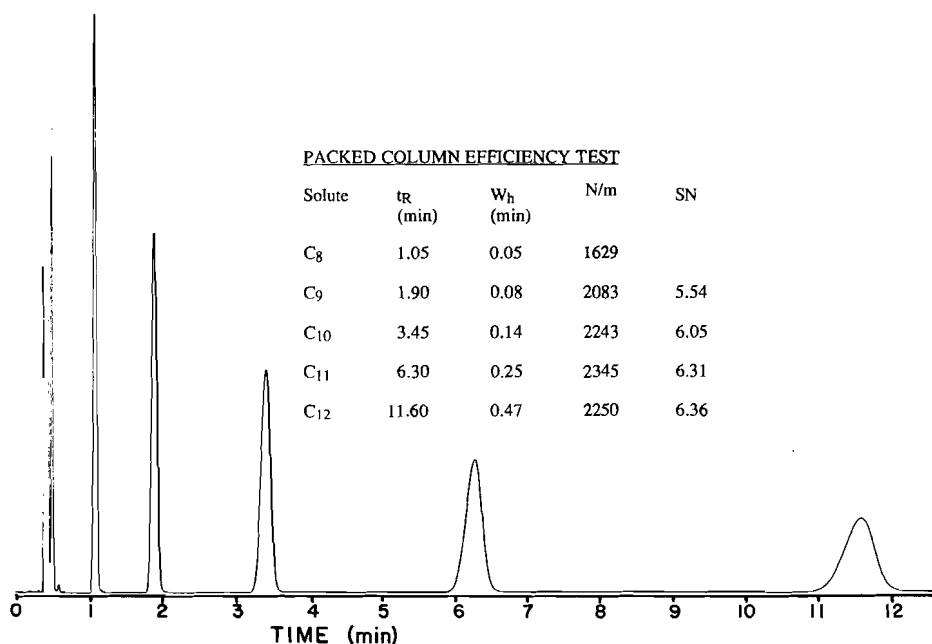


Figure 2.18. Column efficiency test for a 1.5 m x 2 mm I.D. packed column coated with 10% (w/w) OV-101 on Chromosorb P-AW (100-120 mesh) at 100°C with a nitrogen carrier gas flow rate of 30 ml/min. The test sample is a mixture of n-alkanes.

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Chapter 3

Instrumental Aspects of Gas Chromatography

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3.1 INTRODUCTION

The principal function of the gas chromatograph is to provide those conditions required by the column for achieving a separation without adversely affecting its performance in any way. Operation of the column requires a regulated flow of carrier gas; an inlet system to vaporize and mix the sample with the carrier gas; a thermostatted oven to optimize the temperature for the separation; an on-line detector to monitor the separation; and associated electronic components to control and monitor instrument conditions, and to record, manipulate and format the chromatographic data [1-9]. Individual instruments differ mainly in their control functions (whether software based or mechanical), ease of portability (designed for laboratory or field applications), level of automation, and flexibility (number of supported options). Since the early 1990s most instruments have been designed to accommodate the more demanding requirements for open tubular columns with optional modules available to adapt the basic instrument for packed column applications. The primary functions of a typical gas chromatograph can be broken down into pneumatic, thermal and electronic system components as illustrated in Figure 3.1.

3.2 PNEUMATIC SYSTEMS

Gas supplies are required for the carrier gas, and depending on the instrument configuration, perhaps also for the detector, for operating pneumatic controls such as

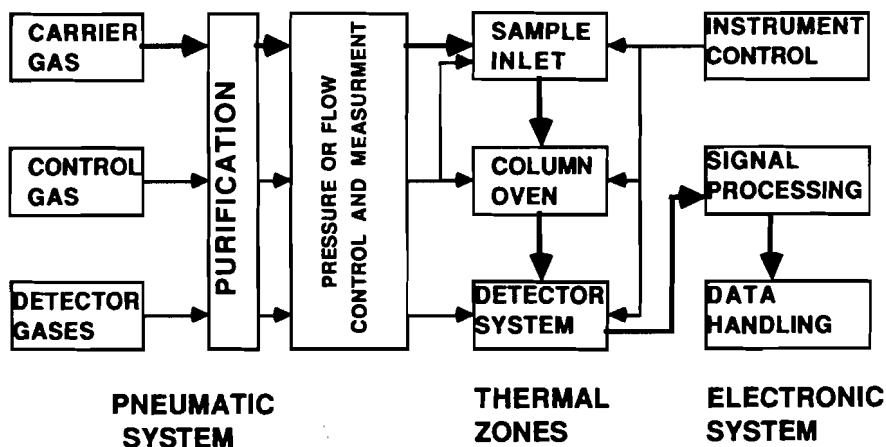


Figure 3.1. Schematic diagram of the principal components of a gas chromatograph. The bold line shows the path taken by sample and carrier gas resulting in the production of a chromatogram. The thin lines represent support and control functions.

switching valves and for providing automatic cool-down by opening the oven door. Gases are normally provided from pressurized cylinders, although compact generators capable of providing air or nitrogen filtered from the atmosphere and hydrogen electrolytically from water are becoming increasingly popular. Each gas cylinder is fitted with a two-stage regulator for coarse pressure and flow control. To minimize contamination high purity gases are used combined with additional chemical and/or catalytic gas purifying devices to maintain a contamination level at or below 1 ppm [10,11]. The carrier gas flow is directed through a particle filter and charcoal and molecular sieve traps to remove low molecular weight hydrocarbons and moisture, and then through an additional chemical trap to remove oxygen. Oxygen causes degradation of some stationary phases, shortens filament lifetime for thermal conductivity detectors and yields unstable baselines with the electron-capture detector. Water shares some of the same properties as well as being a strong deactivating agent that can cause poor reproducibility of retention times in gas-solid chromatography. High purity gases specified for gas chromatography are available and some analysts prefer to use these gases and dispense with rigorous gas clean-up procedures. Support gases for detectors and valve operation, etc., usually require less intensive purification depending on how they are used. Bartram [12] has provided a comprehensive guide to suitable gas management systems for gas chromatographs recommended to anyone involved in the commissioning a new instrument or establishing a gas chromatography laboratory. A suitable plumbing diagram for a single gas chromatograph using pressurized cylinders or gas generators is shown in Figure 3.2.

Pure air generators use laboratory air that is compressed and filtered to remove particle matter, oil and water and a catalytic reactor to remove hydrocarbons (total

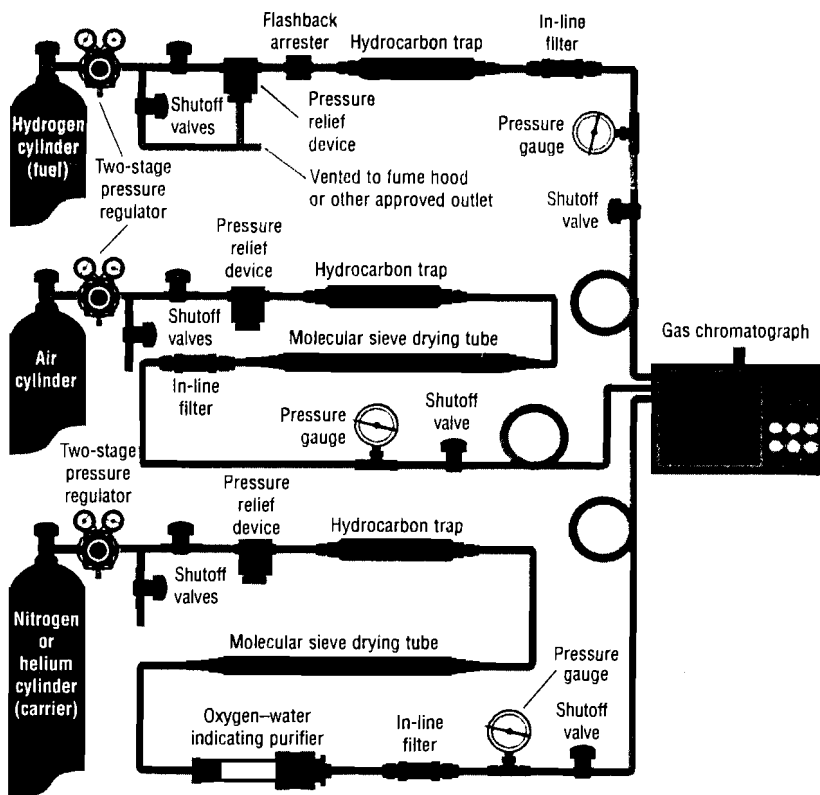


Figure 3.2. Schematic plumbing diagram for a single gas chromatograph with a flame ionization detector using (A) pressurized gas cylinders or (B) gas generators. (From ref. [12]; ©Advanstar Communications Inc.).

hydrocarbons < 0.1 ppm). Pure nitrogen generators use laboratory compressed air, processed to remove general contaminants, from which nitrogen is isolated by selective permeation through polymeric hollow fiber membranes. The purified nitrogen typically contains about 0.5 ppm oxygen, < 0.5 ppm water vapor and < 2 ppb total hydrocarbons. Pure hydrogen (99.999 %) is generated electrolytically from laboratory deionized water using a solid polymer electrolyte and is stored in a small reservoir. Generators are available to provide sufficient gases for single or multiple gas chromatographs at the required operating pressure and flow requirements.

The carrier gas and support gases then enter the pneumatic section of the gas chromatograph, in which pressure regulators, flow controllers and, perhaps, additional gas purifying traps and particle filters are housed in a thermostatted housing to minimize drift. The pressure regulators for the carrier gas are usually of the metal diaphragm type to minimize contamination of the carrier gas with organic impurities and to minimize

the ingress of atmospheric air. Fuel gases required by flame-based detectors need only be coarsely controlled. Common arrangements include a pressure regulator combined with either a calibrated restrictor (snubber) or needle valve. In most early instruments flow control was widely used for packed columns while pressure control was more common for open tubular columns. With a pressure-regulated system poor retention reproducibility due to change in the column backpressure over time and from variations in atmospheric pressure can be a problem. In addition, during temperature program separations the carrier gas velocity will decline with increasing temperature primarily due to increasing carrier gas viscosity. This may cause lower efficiency if the carrier gas velocity becomes too slow towards the end of the temperature program, baseline drift, and changes in response factors for concentration sensitive detectors. The use of makeup gas at the column exit with open tubular columns can improve the stability of concentration sensitive detectors, although sensitivity is reduced by sample dilution.

Since the early 1990s electronic pneumatic control devices have become increasingly popular and are common on all modern keyboard controlled instruments [13-17]. Electronic pressure control uses a solid-state pressure sensor and an electronically controlled proportional valve to achieve precise closed loop control of the column inlet pressure. Electronic pressure programming allows constant mass flow of the carrier gas during temperature programmed operation. The sensing system uses a bypass tube with a heater situated at the center. High precision temperature sensors are located equidistant upstream and downstream of the heater. Gas flow results in a temperature difference between the sensors that is proportional to the mass flow and specific heat of the gas. The voltage generated from the differential sensor is compared to a set voltage for the desired flow and the difference used to operate the proportional valve controlling the gas flow through the main flow channel. The use of sensors and valves allows operation at constant mass flow independent of the column flow resistance, constant pressure, and pressure program modes under full software control. Standard pressure transducers can measure column pressure to about 0.1 or 0.25 p.s.i. Typical electronic flow controllers with a range of 1-500 ml/min are capable of an accuracy of $\pm 1\text{-}2\%$ full scale and a repeatability of the set point of $\pm 0.2\%$ or better.

Simplified diagrams of a mechanical pressure regulator and a flow controller of the diaphragm type are shown in Figure 3.3 [2-4]. The pressure regulator maintains a constant pressure drop across an orifice by adjusting the flow of gas through the orifice. The set point pressure is increased by increasing the tension on the spring by means of a threaded screw, which in turn depresses the diaphragm and increases flow through the orifice. An increase in the backpressure at the outlet will act on the diaphragm to reduce flow at the orifice and maintain a constant pressure differential across the orifice. The flow controller functions as a differential pressure regulator. At a constant inlet pressure and temperature it will deliver a constant mass flow that is independent of the system backpressure, P_4 in Figure 3.3. For a given set point the mass flow rate through the orifice depends on the pressure differential ($P_2 - P_3$) pushing the diaphragm down and the opposing force due to tension in the spring. Any change in the downstream pressure, P_4 , unbalances the diaphragm that controls the opening of the variable orifice

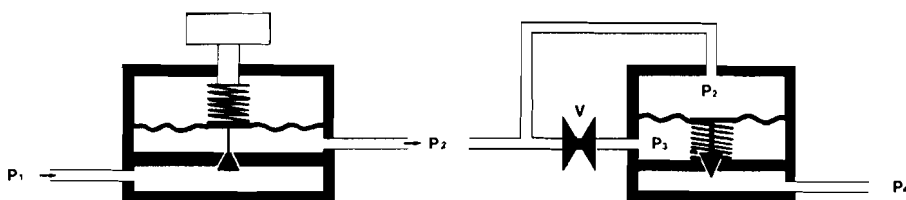


Figure 3.3. Schematic diagram of a pressure regulator and mechanical mass flow controller. P_1 and P_2 are the inlet and outlet pressure of the pressure regulator, respectively. P_2 , P_3 and P_4 are the inlet, reference, and column inlet pressure for the flow controller and V is a needle valve used to set P_3 to the desired value.

to maintain a constant pressure differential across the needle valve, V . This maintains a constant mass flow of gas through the flow controller.

3.3 THERMAL ZONES

The column oven is essentially an insulated box of sufficient size to allow comfortable installation of the longest columns and any accessory equipment normally used. The oven is heated by electrical heating elements arranged around a powerful circulatory fan. Microprocessors, sensors and proportional heating networks are used to maintain a stable isothermal temperature and to control the initial temperature lag, the linearity of the program rate, and the final temperature overshoot in temperature programmed operation. Temperature uniformity depends on the geometry of the oven, the location of the heating elements and sensor, and the pattern of air circulation. A temperature stability of $\pm 0.2^\circ\text{C}$ in time and $\pm 1.0^\circ\text{C}$ in space are minimal requirements. The difference in the set point temperatures between instruments is usually greater than the precision of the set point for each instrument. Poor column temperature stability was identified as a source of peak distortion or splitting with open tubular columns [18,19]. In severe cases Christmas-tree-like peaks are observed. These arise from the oscillation of the solute distribution constants in response to the influence of the fluctuating oven temperature. The thin walls of fused-silica capillary columns rapidly transmit temperature fluctuations from the oven to the phase boundary, allowing little time for cross-wall thermal averaging to dampen the oscillations.

Separations in gas chromatography are carried out at temperatures from about -100°C to 450°C . The temperature operating range for standard chromatographic ovens is from about 40°C to 350°C . Purpose-built instruments are usually required for high temperature operation between 350°C and 450°C . Subambient temperature operation using the boil over vapors from liquid nitrogen or carbon dioxide for cooling is available as an option for standard instruments. The oven temperature is adjusted using an electrically controlled solenoid valve to pulse coolant into the oven where it is mixed with air and then circulated at high velocity. Typical linear temperature program rates are 0.1 to $50^\circ\text{C}/\text{min}$, selectable in incremental steps. Rapid cool down of the column oven is

achieved by automated opening of the oven door or flaps to allow heat dissipation. The power supply and the thermal mass of the oven limit the actual heat-up and cool-down rates. For fast gas chromatography heating and cooling rates much higher than those specified for normal operation are required, for example 1000°C/min, necessitating a different temperature control mechanism than provided by forced air convection ovens. Resistive heating devices using columns with an external conductive coating, a metal sheath around a standard column (a tube within a tube configuration), or a wire adjacent to the column are the favored options [20,21].

Other thermal zones, which should be thermostatted separately from the column oven, include the injector and detector modules. These are generally insulated metal blocks fitted with cartridge heaters and controlled by sensors located in a feedback loop with the power supply. Detector blocks are usually maintained at a temperature selected to minimize detector contamination and to optimize the detector response to different sample types. The requirements for injectors may be different depending on their design, and may include provision for temperature programmed operation.

3.4 SAMPLE HANDLING DEVICES

Gas chromatography is used to separate gases, vapors of volatile compounds supported in a gas matrix, liquids, and solids. Solid samples are usually dissolved in a suitable solvent and handled as for liquid samples. Solids (or liquids) dissolved in supercritical fluids are handled similar to liquids with decompression of the fluid in the injector. Insoluble solids can be handled by direct injection using modified liquid sample inlets [22,23]. Samples are encapsulated in glass capillaries and then pushed or dropped into a hot splitless injector and crushed by a mechanical device releasing the volatile components of the sample. This form of injection is widely used in the analysis of insect body parts and secretions where the trace volatiles of interest might be hidden in the solvent front using conventional injection techniques [24]. A new approach to injecting complex samples uses special disposable microvials heated in an injector to evaporate volatile components from the sample leaving involatile residues in the microvial, which is replaced after every injection [25,26]. Insoluble solids can also be analyzed indirectly by separation of the decomposition products generated by controlled pyrolysis. Compounds in the vapor phase are often separated after concentration on solid sorbents or cryotrap followed by rapid heating to release the organic vapors into a column interface or conventional liquid injector. Thermal desorption interfaces are widely used for the analysis of trace organic volatiles in air or generated by headspace and gas purging sampling devices.

3.4.1 Microsyringes

The most common method of introducing liquid samples into a gas chromatographic inlet is by means of a microsyringe. Typically, this consists of a calibrated glass barrel

Table 3.1
Syringe handling techniques for injection of liquid samples

Method	Principle
Filled needle	• Sample is taken up into the syringe needle without entering the barrel. Injection is made by placing the syringe needle into the injection zone. No mechanical movement of the plunger is involved and the sample leaves the needle by evaporation.
Cold needle	• Sample is drawn into the syringe barrel so that an empty syringe needle is inserted into the injection zone. Immediately the sample is injected by depressing the plunger. Sample remaining in the syringe needle leaves by evaporation.
Hot needle	• Injection follows the general procedure described for the cold needle method except that prior to depressing the plunger the needle is allowed to heat up in the injector for 3-5 seconds.
Solvent flush	• A solvent plug is drawn up by the syringe ahead of the sample. The solvent and sample may or may not be separated by an air barrier. The injection is usually made as indicated in the cold needle method. The solvent is used to push the sample out of the syringe.
Air flush	• As for solvent flush, except that an air plug is used rather than a solvent plug.

with a close fitting metal plunger, which is used to dispense a chosen volume of sample by displacement through the syringe needle. Gas-tight syringes, some with a valve mechanism to close the passageway to the needle, are available for injecting gases and vapors. These generally have Teflon-tipped plungers for improved sealing of the plunger with the syringe barrel against the backpressure created by the inlet. Syringes are excellent for qualitative analysis of gases and vapors, and may give reliable quantitative results for large sample volumes. An internal standard is generally required for quantitative analysis of small sample volumes.

Although microsyringes are easy to use and considered indispensable for the injection of liquids, they do result in certain difficulties. The accuracy of quantitative injection depends on the rate of sample introduction, syringe dead volume, heating of the syringe needle by the injector and sample handling techniques. The most common syringe injection techniques are summarized in Table 3.1. Packed columns accommodate relatively large sample volumes (e.g. 1-5 μl) and are forgiving of poor injection techniques. Most of the methods in Table 3.1 will work quite well, with the solvent flush method being the most popular. The injection of comparatively small volumes, typically 0.1 to 2.0 μl , is more common for open tubular columns. In this volume range needle dead volumes, sample adhering to the outside wall of the needle and backflushing of sample past the plunger can represent significant contributions to poor injection precision [27-31]. For vapor samples adsorption on the syringe barrel can be a substantial source of low sample recovery and can be detected (corrected) by the method of successive re-injection [32]. Adsorption or catalytic decomposition of labile substances by the syringe needle can be a problem for some compounds using hot vaporizing injectors [33].

The absolute injection volume can be difficult to determine when microsyringes are used with hot vaporizing injectors. At the time of injection the sample volume delivered

to the inlet is equivalent to the calibrated amount determined by the graduations on the syringe barrel plus some fraction of the sample volume retained in the needle. The correction for the needle volume is difficult to determine, since it will depend on the column inlet temperature and pressure. After injection, estimating the non-injected sample volume by drawing the remaining sample up into the syringe barrel is subject to error due to loss of vapors through the syringe needle while equilibration to ambient conditions occurs.

Discrimination of sample components based on volatility differences is a common problem encountered when using microsyringes for sample introduction into hot vaporizing injectors coupled to open tubular columns. The sample leaves the syringe and enters the vaporizer as a stream of droplets, formed by the movement of the plunger and by evaporation of the remaining sample from the syringe needle. It is at this evaporation stage that discrimination is most likely to occur; the solvent and more volatile sample components distill from the syringe needle at a greater rate than the less volatile components. Consequently, the sample reaching the column is not identical in composition with the original sample solution; it contains more of the most volatile and less of the least volatile components of the sample. Rapid injection using autosamplers and injection devices that allow introduction of the sample at temperatures below the solvent boiling point are the best solution to this problem. Using the "hot needle" and "solvent flush" methods indicated in Table 3.1 also reduce discrimination.

3.4.2 Gas Sampling Valves

Rotary valves with internal or external sample loops, and to a lesser extent piston or membrane valves, are generally preferred for introducing gases and volatile organic compounds into all column types [2,34]. Rotary valves with 6, 8 or more connection ports, operated manually or automated through electronic or pneumatic controllers, are commonly used in laboratory instruments for sample introduction as well as column switching and applications in multidimensional gas chromatography. The gas sample to be injected is isolated in a sample loop of defined volume at a known temperature and pressure (usually ambient). Subsequent rotation of the valve body causes the carrier gas to sweep the entire contents of the sample loop onto the column. External sample loops with defined volumes in the range 0.5 μ l to 5 ml are commonly used. Valves with internal sample loops are available for handling small sample volumes of 0.2 to 5 μ l. Multiport valves with two or more sample loops can be used for simultaneous sampling of several sources or for continuous sampling of a single source by storing individual samples in different loops for sequential analysis at a later time.

Gas sampling valves are usually mounted in the column oven, in a separately heated adjacent oven, or external to the column oven and connected to the column by a short length of capillary tubing. For the most accurate work it is generally recommended that the injection valve is thermostatted in its own oven. Since the valve materials are in contact with the sample they must be chemically and physically inert, free from outgassing products, gas tight at all temperatures, and operate without lubricants.

They are typically constructed from stainless steel bodies with fluorocarbon filled, crosslinked, polyimide resin rotors. Other materials are available for exceptional sample or environmental operating conditions. Valves constructed from different materials are capable of leak-free operation and a reasonable service life over the temperature range from -198 to 350°C. For corrosive samples a system based on a Deans' switch, which allows transfer of a known sample volume to the carrier gas stream without contact with the valve body or sample loop, may be beneficial [35]. Pneumatically actuated rotary valves with small internal loops (60 nl) have been used as sample introduction devices for narrow bore capillary columns in high-speed gas chromatography [36,37]. Combining a septum injector upstream of a gas-sampling valve in a closed-loop configuration provides a convenient method of generating a long-lived gas vapor mixture for physicochemical studies requiring multiple injections [38]. Rotary valves are also used for large volume sample introduction in on-column injection and as an interface for coupling liquid chromatography to gas chromatography.

3.5 SAMPLE INLETS

The sample inlet provides the means by which the sample is vaporized and mixed with carrier gas prior to the start of the separation. These processes should be achieved without reduction of the separation potential of the column; in the absence of thermal degradation, adsorption or rearrangement of sample components; without discrimination of sample components by boiling point, polarity or molecular weight; and with quantitative recovery for both trace and major sample components. It is also preferable that changes in the column operating conditions should not affect the sampling process. It is difficult to meet all of these requirements with a single inlet design for the wide range of sample and column types encountered in gas chromatography. The absence of a universal inlet has resulted in the development of a number of specialized inlet systems required to manage the full range of gas chromatographic applications [39-43].

3.5.1 Packed Column

For packed columns injection of the sample in solution is made with a microsyringe through a silicone rubber septum into a glass liner or the front portion of the column, which are heated and continuously swept by carrier gas, Figure 3.4. When injection is made in the on-column mode, the column is pushed right up to the septum area and the column end within the injector is packed with glass wool. Ideally, the tip of the syringe needle should penetrate the glass wool filling and just reach the surface of the column packing. For flash vaporization the sample is injected into a low dead volume, glass-lined chamber, mixed with carrier gas, and flushed directly onto the column. Whichever technique is used, the injector must have sufficient thermal mass to rapidly vaporize the sample. The incoming carrier gas is usually preheated by directing its flow through

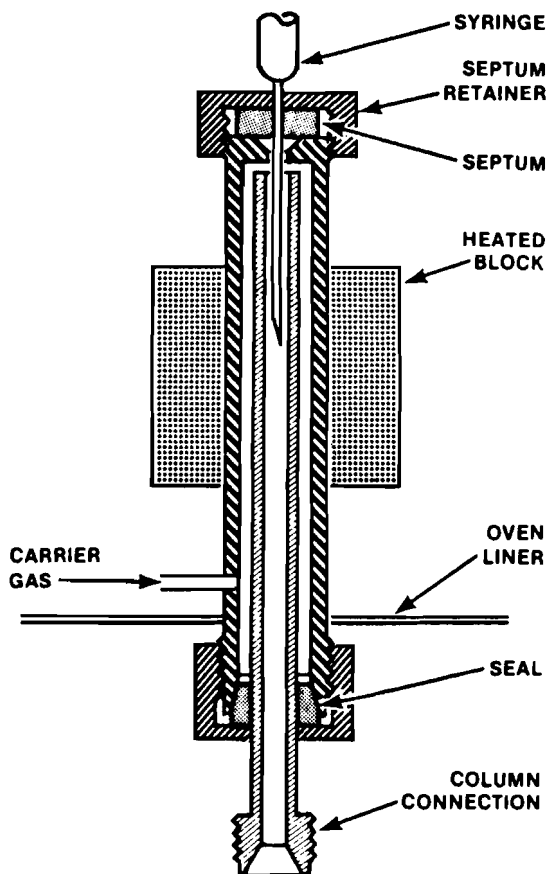


Figure 3.4. An example of a flash vaporization inlet for packed column gas chromatography.

a section of the injector block to avoid possible condensation of the vaporized sample upon mixing with the cool carrier gas. The temperature of the injector block should be selectable over the range 25 – 400°C. Typically, it is set 50°C higher than the maximum column temperature used for the separation unless sample instability dictates otherwise. Because high injection temperatures are frequently used, septum bleed may give rise to an unstable baseline or the appearance of ghost peaks in the chromatogram. Various solutions to this problem are available; low-bleed septa with good resealability, a finned septum holder that allows cooling of the septum, a septum purge device, or an airlock can be used. Several arrangements can be used for the septum purge, but in all cases a portion of the carrier gas, or an auxiliary gas, is forced to flow across the face of the septum and out through an adjacent orifice

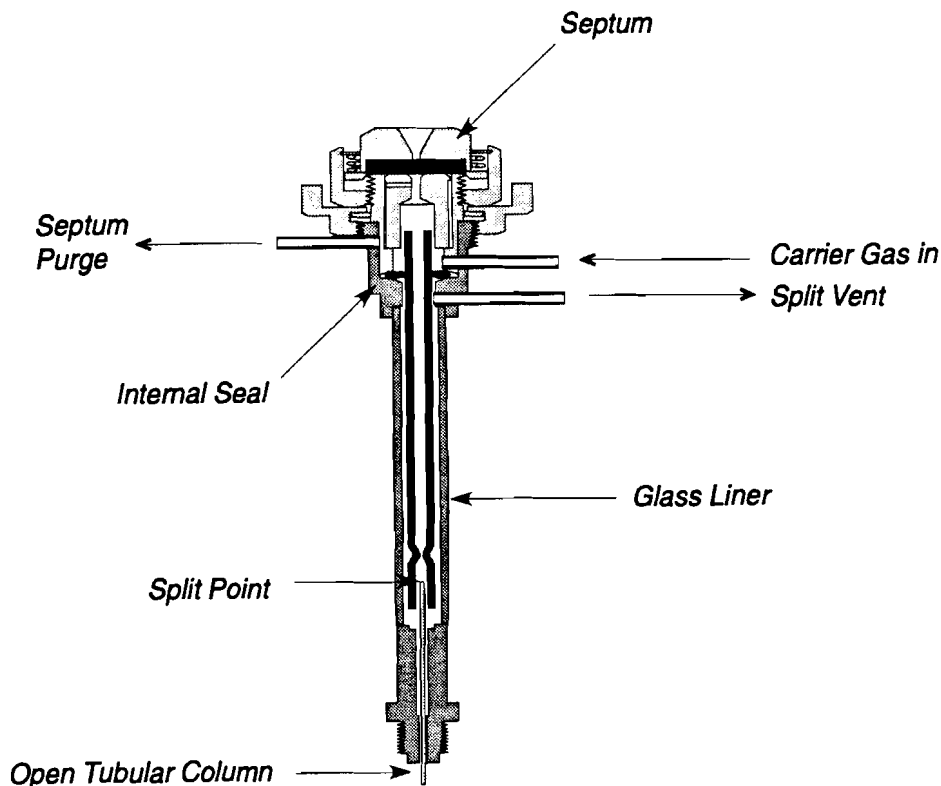


Figure 3.5. Schematic diagram of a hot split/splitless injector for open tubular columns. (From ref. [50]; ©Perkin-Elmer Co.).

3.5.2 Hot Split/Splitless

For many qualitative applications classical split injection is the most convenient sampling method. It allows injection of mixtures virtually independent of the choice of solvent for the sample, at any column temperature, with little risk of disturbing solvent effects or band broadening in time due to slow sample transfer from the injector to the column [39,41-50]. The classical hot split injector, Figure 3.5, is really an isothermal vaporization injector, in which the evaporated sample is mixed with carrier gas and divided between two streams of different flow, one entering the column (carrier gas flow) and the other vented to the outside (split flow). The vaporization chamber is usually constructed from a stainless steel tube lined with a removable glass liner to minimize sample contact with hot reactive metal surfaces. The gas flow to the inlet is controlled by a forward-pressure or backpressure configuration [15,17,41,49]. In the forward-pressure configuration the inlet pressure is controlled by a pressure regulator in front of the inlet and the split flow by an on/off solenoid valve and a fixed restrictor or

needle valve at the split vent exit from the inlet. The backpressure configuration uses a flow controller to establish the total flow of carrier gas to the inlet and a backpressure regulator in the split vent line to control the inlet pressure. The liquid sample is introduced into the vaporization chamber by syringe through a septum or airlock where it evaporates and mixes with the gas stream. A portion of the carrier gas and sample mixture then passes into the column inlet; the majority is routed out of the system through the split exit. An adsorption filter and/or buffer volume in the splitter line is used to prevent condensation of the sample vapors in the split line. An auxiliary flow of gas is used to purge septum bleed products and contaminants away from the vaporization chamber. Appropriate column loads are usually achieved by injecting sample volumes of 0.2 to 2.0 μl with split ratios between 1:10 to 1:1000; the normal range of split ratios being 1:20 to 1:200.

The introduction of gas or vapor samples does not normally affect the preset split ratio. For liquid samples the instantaneous split ratio depends in a complex manner on several parameters, including the sample volatility range, sample solvent, volume injected, inlet pressure, syringe handling technique, and the injector temperature and volume. For mixtures containing sample components of unequal volatility split injection discriminates against the less volatile sample components due to selective evaporation from the syringe needle (section 3.4.1) and from incomplete sample vaporization and inhomogeneous mixing of sample vapors with the carrier gas in the vaporization chamber. Sample evaporation generates an instantaneous pressure pulse and rapid change in the viscosity of the carrier gas sample mixture, altering the flow of gas between the column and split line in an irreproducible manner. The residence time of the sample in the vaporization chamber is insufficient for the transfer of sufficient heat to the sample to complete the vaporization process. In most cases the sample arrives at the split point only partially evaporated as a mixture of vapor and droplets of various sizes. The sample components are unlikely to be evenly distributed between the vapor and liquid phase, the latter being split to different extents and resulting in discrimination. Discrimination is the principal cause of difficulties in quantitative analysis. Control of all aspects of the injection process is very important for acceptable repeatability and some guidelines for performing hot (classical) split injection are summarized in Table 3.2.

According to the explosion-like evaporation model, the amount of sample entering the column depends on the magnitude of the pressure wave, the time taken for the pressure wave to reach a maximum and the column temperature [44-47]. The pressure wave, caused by sample evaporation, fills the capillary column with a portion of the sample vapor followed by a period when the pressure falls back to normal. During this second period little sample enters the column and most of the sample vapor is vented through the split exit. The preset split ratio influences the sample-split ratio by its affect on the magnitude of the pressure wave and its duration. Column temperature influences the split ratio through sample condensation. Condensation greatly reduces the volume of sample vapor in the cooled column inlet, creating a zone of reduced pressure that sucks in further amounts of sample vapor. This causes a decrease in the

Table 3.2

Factors affecting the repeatability of liquid injection using classical hot split injectors

Parameter	Considerations
Volume	The magnitude and duration of the pressure wave depends on the sample size. <i>Reproduce the sample volume precisely for all injections.</i>
Solvent	The solvent identity and boiling point at the inlet temperature and pressure affects the volume of vapor produced and hence the pressure wave. Solvent volatility can also influence the distribution of sample between the vapor and droplet phases. The same sample amount dissolved in different solvents may produce different peak areas. <i>All samples should be dissolved in the same solvent.</i>
Syringe handling	Slow movement of the plunger can almost eliminate the pressure wave but results in enhanced discrimination of sample components. <i>Rapid injection using the hot needle or solvent flush method is preferred. Autosamplers capable of high speed and reproducible injection times provide improved performance.</i>
Release position	Maximum amount of sample enters the column when the sample is released near the column inlet. This will depend on the design of the injector and the length of the syringe needle. <i>Reproduce the needle penetration length precisely.</i>
Liner packing	A light packing of silanized glass or quartz wool improves sample evaporation and hinders involatile residues from entering the column. It can reduce discrimination and improve the reproducibility of peak areas. Packing materials promote decomposition of labile compounds and can retain components of low volatility. Glass wool and other packing materials may improve or worsen quantitative aspects of split injection. <i>Precaution advised as the outcome is unpredictable in many cases.</i>
Column temperature	Important because of the condensation effect when the column temperature is close to the boiling point of the solvent or principal sample component. <i>The initial column temperature should be fixed and constant.</i>
Standards	<i>Internal standards are recommended for quantitative analysis.</i> If standard additions are used for calibration all parameters listed in this table must be held constant.

split ratio (i.e. an increase in observed peak areas) as the column temperature is reduced and is particularly important at column temperatures near the solvent boiling point. At column temperatures 50 -80°C below the solvent boiling point, condensation is virtually complete and further decreases in column temperature have little effect.

Splitless injection uses a similar injector design to split injection but is more suitable for quantitative analysis of trace components in dirty samples, such as biological and environmental extracts [41,49-59]. Conversion of a split to a splitless injector usually requires no more than the installation of a different liner and the interruption of the split flow at the start of the injection using a solenoid valve located in the vent line. The split flow is restarted only at the end of the sampling period. Since the flow of gas through the vaporization chamber is normally the same as the optimum carrier gas flow for the column the transport of sample vapors to the column is relatively slow. During the initial rapid evaporation of the sample there is minimal transfer of vapors into the

column. Consequently, the volume of the injection liner must be large enough to hold the entire volume of vapor produced by the evaporated sample. If the volume of the vaporization chamber is too small, sample vapors will be lost by backflushing through the septum purge exit or by deposition in the carrier gas lines. In addition, the sample should be released near the bottom of the chamber, close to the column, so that it fills with vapor from the bottom up, displacing the carrier gas backwards. A liner volume of about 1 ml is sufficient to hold up to about 0.5 - 2 μ l of vaporized solvent, which should be injected rapidly to minimize discrimination resulting from selective evaporation of the sample from the syringe needle. Splitless inlets equipped with electronic pressure control allow high inlet flows at the start of injection, followed by a rapid reduction of pressure to a value required to provide the desired column flow rate [56,57,60]. Higher flow rates at the time of injection cause the sample to be swept more rapidly into the column while simultaneously reducing the expansion volume. Larger sample volumes can be introduced with a standard liner, up to 5 μ l, but sample focusing mechanisms reliant on solvent effects are less effective, particularly for early eluting compounds.

Sample transfer to the column by splitless injection should be virtually complete but requires a comparatively long transfer time, from several seconds up to a few minutes, relying on cold trapping and/or solvent effects to refocus the sample at the column inlet. As a rough guide, the sample transfer time will be equivalent to about twice the time required by the carrier gas to sweep out the volume of the vaporization chamber. Complete sample transfer is difficult to achieve, since the sample vapors are continually diluted with carrier gas, and some sample vapors accumulate in areas poorly swept by the carrier gas. At the end of the sample transfer period, the split flow is re-established to purge the inlet of remaining solvent vapors. If the split flow is started too soon, sample will be lost; if too late, the solvent peak will trail into the chromatogram. When the conditions are correct there will be no significant sample loss and the solvent peak will be rectangular.

The attractive features of splitless injection techniques are that they allow the analysis of dilute samples without preconcentration (trace analysis) and the analysis of dirty samples, since the injector is easily dismantled for cleaning. Success with individual samples, however, depends on the selection of experimental variables of which the most important: sample size, sample solvent, syringe position, sampling time, initial column temperature, injection temperature and carrier gas flow rate, often must be optimized by trial and error. These conditions, once established, are not necessarily transferable to another splitless injector of a different design. Also, the absolute accuracy of retention times in splitless injection is generally less than that found for split injection. For splitless injection the reproducibility of retention times depends not only on chromatographic interactions but also on the reproducibility of the sampling period and the evaporation time of the solvent in the column inlet, if solvent effects (section 3.5.6.2) are employed. The choice of solvent, volume injected and the constancy of thermal zones will all influence retention time precision beyond those for split injection. For quantitative analysis the precision of repeated sample injections is normally acceptable but the method is subject to numerous systematic errors that may

affect accuracy. Internal standards are usually preferred to external standards to improve accuracy. A liner packed with a light plug of silanized glass or quartz wool may reduce matrix effects and harmonize evaporation rates but also promote the decomposition of labile solutes and enhance other problems.

Hot vaporizing injectors are subject to several matrix problems in addition to contamination by involatile residues [61-65]. The presence of involatile matrix components may change the evaporation rate of the sample and possibly result in incomplete evaporation. The droplets formed by involatile components may completely retain or only slowly release certain solutes compared to a clean sample. In a split injector, the presence of long-lived droplets may change the intensity and duration of the pressure wave, and with it the split ratio. Compared to a clean sample the presence of involatile material may reduce the amount of the most volatile components while allowing about the same amount of the least volatile components to enter the column. Matrix induced response enhancement was observed during hot splitless injection of some organophosphate, carbamate and organochlorine compounds in the presence of sample matrix. The vaporization chamber contains active sites associated with glass surfaces and deposits of involatile residues remaining from previously analyzed samples. For real samples the matrix coextractants compete with the analytes for active sites resulting in a larger transfer of analytes to the column. When standards prepared in matrix-free solvent are used for calibration an overestimate of the analyte concentration in matrix-protracted (real) samples is obtained. Matrix-matched standards for calibration or pulsed splitless injection are used to solve this problem. The former case requires a sufficient supply of analyte-free sample matrix and the latter relies upon the shorter residence time of the sample in the vaporization chamber to minimize interactions with active sites.

3.5.3 Direct

Direct injection is used with wide bore open tubular columns (≥ 0.32 mm) that operate with flow rates in the range of 5 to 15 ml/min. Direct injection uses a thermostatted hot vaporization chamber with a direct connection between the liner and the column. Typically, a double gooseneck liner is used with the column fixed to the constriction at the bottom end (the seal is usually made between the polyimide outer coating of the column and the inner glass surface of the liner). During injection, the syringe needle partially constricts the restriction at the top of the liner. Relatively high flow rates are required to sweep the sample onto the column in a reasonable time. If the retention power of the stationary phase is inadequate to focus sample bands then solvent effects and cold trapping must be employed, in the same manner as splitless injection. One reason for selecting direct injection is its simplicity and minimal optimization compared to splitless injection. Since it is used with columns of modest efficiency many minor transgressions of technique go unnoticed or are tolerated. On the other hand, it is subject to all the matrix effects and syringe handling problems observed for splitless injection. Injection volumes are usually in the range 2 – 8 μ l with removal of the syringe

needle from the vaporization chamber delayed until the pressure wave has dissipated to avoid backflushing of sample vapors into the septum purge and carrier gas lines. A hot split/splitless injector can be converted to direct injector by changing the liner and turning off the split vent. Using a liner with a single restriction at the top end where the column seal is made allows the injector to be converted into a hot on-column injector. In this case the restriction acts as a base for the column seal and a needle guide to direct the syringe needle into the column or a retention gap connected to the column.

3.5.4 Programmed Temperature Vaporizer

The programmed temperature vaporizer injector (PTV), Figure 3.6, provides technical solutions to some of the problems identified as the primary concerns in the use of classical hot split/splitless and direct injectors [39,41,66-73]. Early versions of this injector were developed to allow large volume injections with solvent elimination and to avoid discrimination arising from selective sample evaporation from the syringe needle. The liquid sample is introduced by syringe into the vaporization chamber, which is maintained at a temperature below the solvent boiling point. The PTV vaporization chamber is about one-tenth the size and of lower thermal mass than chambers employed for classical hot split/splitless injection. This allows the vaporization chamber to be rapidly heated by either direct or indirect resistive heating, using either cartridge heaters or circulated hot air (10-15°C/s). Cooling can be performed using cold air, a Peltier element, or expanding carbon dioxide or liquid nitrogen vapors.

The PTV injector can be used as a split/splitless injector for samples of normal volume, as a large volume injector and as a device for automated liquid or vapor sample introduction when interfaced to a number of sample preparation devices. For normal split injection the sample is introduced at a temperature below the solvent boiling point with the split exit open. Shortly after withdrawal of the syringe needle the injector is rapidly heated to a temperature sufficiently high to ensure rapid evaporation of the least volatile sample components. Relatively slow sample evaporation in the injector aided by the presence of some packing material, usually glass wool, avoids the formation of aerosols, which are formed in hot vaporizing injectors. Dilution of sample vapors with carrier gas avoids condensation of the solvent in the column inlet. Also, since the sample is introduced into a cold injector there is no pressure wave. Discrimination effects, that are common for hot vaporizing injectors, are virtually eliminated and the sample split ratio and split flow ratio are similar. For cold splitless injection the sample is introduced into the vaporizing chamber at a temperature close to the solvent boiling point with the split vent closed. A few seconds after injection the injector is rapidly heated to the temperature required to complete the transfer of vapors into the column (30-90 s), the column temperature program is started, and the vaporizer is purged by opening the split vent to exhaust any solvent residues. Similar to hot splitless injection, cold trapping and solvent effects are employed as refocusing mechanisms. Since sample transfer takes place in two steps: transfer of solvent and volatiles followed by transfer of less volatile components at a later

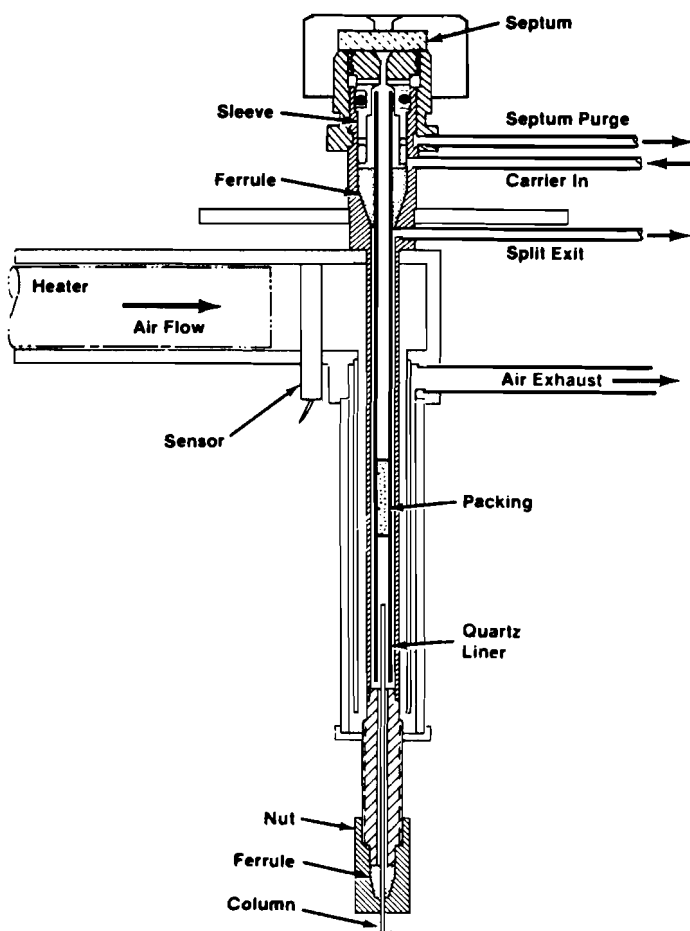


Figure 3.6. Schematic diagram of a PTV type injector. (From ref. [66]; ©Wiley-VCH).

time, these refocusing mechanisms are generally most important for the separation of the volatile sample components. The precision and accuracy of the PTV techniques are generally superior to those of the classical hot split and splitless techniques and approach those obtained by cold on-column injection. The latter remains the preferred injection technique for clean samples, but PTV injection is better suited for dirty samples (i.e. those contaminated by involatile impurities) and for headspace vapors.

For large volume injection the PTV injector can be operated as a cold split injector with solvent elimination, as a splitless injector with vapor discharge, or as a hot splitless injector using vapor overflow for solvent elimination. In cold split injection, the sample is introduced into the injector at a temperature below the solvent boiling point with the

split vent open. The solvent is concurrently evaporated in the glass insert and swept through the split vent by the carrier gas. After completion of the solvent evaporation, the split vent is closed and the injector rapidly heated to the temperature required to vaporize the sample and transfer it to the column. The maximum volume of sample that can be injected depends on the dimensions of the liner and the amount of packing material, usually glass wool. Other suitable packing materials include PTFE wool and some coated supports when glass wool proves to be too active [74-76]. Large sample volumes can be introduced in several increments, or more elegantly, with a speed-controlled injector. The maximum sample volume is limited to about 20 μl (1.2 mm I. D. liner) or 150 μl (3.4 mm I. D. liner) using a single injection (at-once injection) or about 1 ml using speed-controlled injection. During solvent elimination, cold trapping and solvent effects are responsible for the retention of the analytes in liners packed with glass wool [68]. Cold trapping is sufficient for trapping solutes of intermediate and low volatility. For volatile analytes cold trapping must be supplemented with solvent effects. In this case, it is important that the solvent vent is closed slightly before the last trace of liquid evaporates from the liner. The effectiveness of cold trapping and solvent effects in retaining volatile analytes in the liner is demonstrated in Figure 3.7 for the injection of 100 μl of a hexane solution containing 0.2 $\mu\text{g/ml}$ each of C_8 to C_{30} n-alkanes.

For large volume splitless injection the sample is introduced at a temperature below or close to the pressure corrected solvent boiling point with the split vent closed. Solvent vapors are discharged through the separation column. Compared with the split injection configuration, volatile compounds are trapped in the solvent swollen stationary phase at the column inlet rather than lost through the split vent. Since the flow rate of gas through the vaporization chamber is the same as the carrier gas flow rate, solvent elimination is slow and this method is not widely used. The maximum volume of sample that can be introduced is about 20 – 30 μl .

For hot splitless injection with vapor overflow the sample is injected rapidly into the lower part of the packed liner at a temperature well above the solvent boiling point. Violent evaporation causes most of the expanding solvent vapor and volatile sample components to escape through the septum purge outlet. Solutes of low volatility are retained in a low temperature zone created by the evaporating solvent. After solvent elimination the temperature of the retaining zone rapidly returns to the injector temperature transferring the analytes to the column. Tenax or silanized glass wool are commonly used as packing material to increase the retention power of the liner. Even so, analytes less volatile than nonadecane are difficult to trap quantitatively. This method can also be used with classical hot splitless injectors [74].

The PTV injector is a versatile and increasingly popular sample inlet. Compared to hot split/splitless injection it greatly reduces discrimination of less volatile compounds, minimizes thermal degradation because of the shorter sample residence time at elevated temperatures, and is adaptable to the introduction of a wide range of sample volumes. Its quantitative accuracy is nearly as good as cold on-column injection as well as being more tolerant of involatile matrix components. The PTV is a useful interface for coupling a number of sample preparation methods to gas chromatography such as

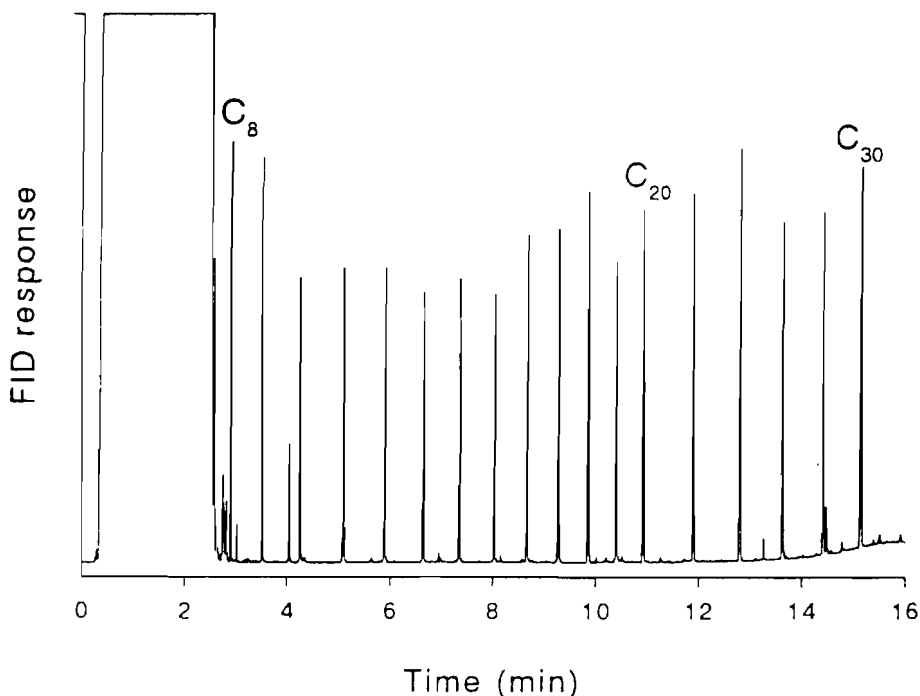


Figure 3.7. Large volume injection of 100 μl of a mixture of n-alkanes of wide volatility using the PTV injector in the cold split solvent elimination mode. The vaporizing chamber was thermostated at 0°C, split flow 250 ml/min with the split vent closed after 2.5 min. For splitless transfer the vaporizing chamber was heated at 4°C/s to 325°C with the purge flow started after 1.5 min. (From ref. [68]; ©Wiley-VCH).

high performance liquid chromatography (LC-GC), supercritical fluid extraction (SFE-GC), solid-phase extraction (SPE-GC), and thermal desorption (TD-GC) [70,71,77]. Replacing the injector liner with a sorbent filled tube allows the PTV injector to be used for integrated sampling and sample introduction for volatile organic compounds in air [78] and for large volume injections of gaseous samples [79]. The PTV injector also has been used as a thermal analyzer and pyrolysis device for solid samples [71,80].

3.5.5 Cold On-Column

Cold on-column injection employs the direct introduction of the sample as a liquid into the oven-thermostatted column inlet or precolumn without prior vaporization in a heated external chamber [40,42,43,69,81]. The sample is subsequently vaporized from the liquid layer formed in the column inlet or precolumn. Cold on-column injection is the only technique in which the composition of the sample introduced into the column is identical to the original sample composition. Discrimination is virtually eliminated and quantification of components of different volatility is facilitated. Sample

decomposition due to thermal and catalytic effects is minimized. On-column injection is easily automated using wide bore precolumns connected to the separation column and is easily adapted to the introduction of large sample volumes using the retention gap technique. On-column injection is not suitable for the introduction of samples containing significant amounts of involatile matrix components that accumulate at the column inlet increasing its retentive power and activity. Samples containing significant concentrations of water (and other reactive components) are problematic because they tend to destroy the deactivation layer of the column rendering it adsorptive. In addition, water and other non-wetting solvents tend to provide weak sample focusing effects resulting in poor peak shapes and retention reproducibility [82].

On-column injection requires special syringes with small external diameter stainless steel or fused-silica needles (since at the point of sample release the needle must reside within the column) or the use of wide diameter precolumns (retention gap) connected to the separation column that are compatible with standard syringe needles. The smallest needles available have an outer diameter of about 0.17 mm and are used with 0.25-mm internal diameter columns. These needles are very fragile and not particularly easy to clean or fill. The 0.23 mm outer diameter (32 gauge) needles are more robust, easier to handle, and are suitable for use with 0.32 mm internal diameter columns. Standard (26 gauge) syringe needles with an external diameter of 0.41-mm outer diameter are compatible with wide bore columns or precolumns and are used in automated injectors.

The injection device must provide a mechanism for guiding the syringe needle into the column, for positioning the needle at the correct height within the column and for sealing the needle passageway, or at least restricting the flow of carrier gas out through the syringe entry port. The length of the syringe needle must be adapted to the injector used; the needle must pass through the injector scaffold and reach at least a few millimeters into the thermostatted column inlet. A number of different methods based on the use of valves, septa, or spring loaded O-ring seals are used for this purpose. A typical injector using a duckbill valve sealing mechanism is shown in Figure 3.8. Circulating air is used in some designs to control the temperature of the needle passageway to avoid solvent evaporation. This is important in high temperature gas chromatography as it allows the oven temperature to be maintained well above the solvent boiling point [83]. Alternatively, the column inlet can be housed in a separate temperature programmable injection oven that can be thermostatted independently of the column oven [1].

On-column injection can be carried out successfully by adhering to a few simple guidelines. Sample volumes of about 0.2-1.5 μl are injected rapidly into the column. For larger volumes up to about 50 μl a precolumn (retention gap) is required to avoid peak distortion by band broadening in space. A retention gap combined with an early vapor exit is suitable for injections up to about 100 μl with larger volumes introduced at a controlled speed using partial concurrent evaporation. For injection of small sample volumes the column temperature, or the thermostatted column inlet, should be maintained at or below the solvent boiling point at the carrier gas inlet pressure. This ensures that the sample is introduced as a liquid that flows into the column inlet or precolumn driven by the carrier gas while avoiding discrimination caused by

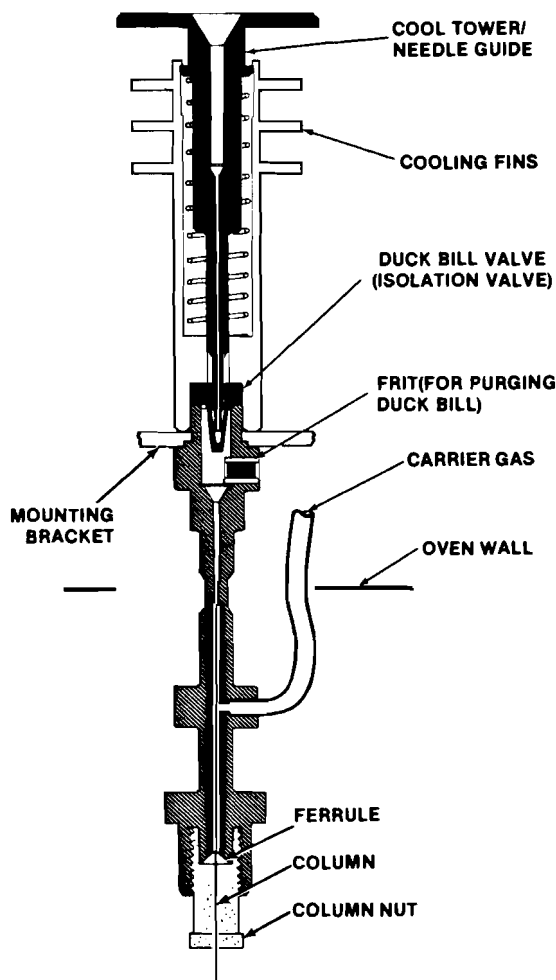


Figure 3.8. Cold-on column injector with a duckbill valve sealing mechanism (©Hewlett-Packard Co.).

sample evaporation from the syringe needle. The liquid spreads as a film on the column wall with gentle evaporation of the solvent starting from the rear to the front with the vapors carried away by the carrier gas. After injection, the column oven should be abruptly raised to the operating temperature after a delay of 30 to 60 seconds to complete solvent evaporation, if the separation temperature is significantly different from the solvent boiling point. Temperature programming is started from either of these two initial temperature settings depending on sample volatility. Quantitative accuracy and repeatability ($< 1\%$ RSD for automated injectors) is excellent using internal standards to correct for differences in volume delivery.

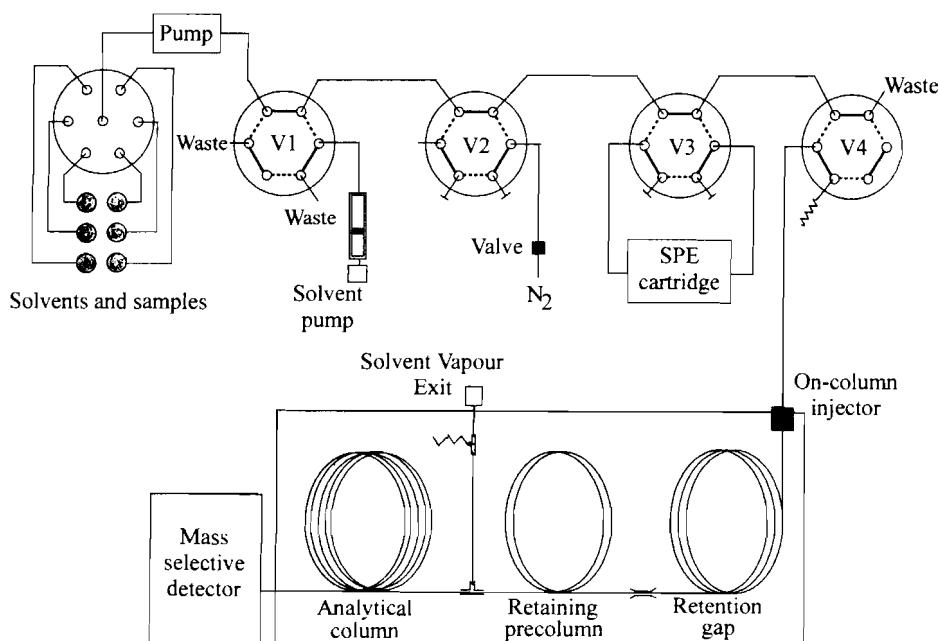


Figure 3.9. Schematic diagram of an automated on-line solid-phase extraction-gas chromatography system. Large volume on-column injection with an early solvent vapor exit and retaining precolumn is used for sample transfer. (From ref. [117]; ©Elsevier).

For large volume injection a retention gap is used with an early solvent vapor exit and optional retaining precolumn [84-90]. Introducing the sample at a controlled speed slightly higher than the solvent evaporation rate at a temperature below the pressure corrected solvent boiling point, guarantees the formation of a solvent film on the wetted surface of the retention gap. Partial concurrent solvent evaporation allows a large fraction of the solvent to be evaporated during the injection and a reduction in the required length of the retention gap, which need only be long enough to retain a fraction of the sample volume. To optimize the injection process the maximum speed of injection, the length of the retention gap, injection temperature and the solvent evaporation rate have to be adjusted so that the solvent film will not reach the stationary phase of the separation column. Solutes of low volatility deposited over the length of the flooded zone are refocused after the completion of solvent evaporation by cold trapping. Many detectors will not tolerate the large volume of solvent vapor produced during injection. These vapors are diverted from the chromatographic system using an early solvent exit located between the retention gap or retaining precolumn and the separation column, as shown in Figure 3.9. Solvent effects are responsible for the retention of volatile analytes. To avoid their loss the solvent vapor exit must be closed just before the last drop of solvent evaporates. For this critical step several approaches are available:

monitoring the effluent leaving the solvent vapor exit with a flame; performing a series of injections with different closure times and estimating the optimum value from these results; monitoring the carrier gas flow into the gas chromatograph; or measuring the temperature with a microthermocouple attached to the precolumn wall close to the vapor exit. Solvent evaporation consumes substantial amounts of energy, which results in cooling by several degrees. Hence passage of the rear end of the flooded zone at a given point is detectable by the temperature drop and the signal generated used to close the vapor exit. During injection and evaporation processes the carrier gas flow rate decreases, mainly because of the presence of solvent vapors in the gas phase, and the completion of the evaporation process is indicated by a steep increase in the carrier gas flow rate to its original value. Monitoring the carrier gas flow provides an alternative approach for automation of the closure of the solvent vapor exit. Up to about 1 ml of sample can be transferred to a 10 m x 0.53 mm I.D. retention gap with partial concurrent solvent evaporation eliminating 75% of the solvent as vapor through the solvent vapor exit.

3.5.6 Focusing Mechanisms

During splitless injection the sample enters the column over a period of time considerably longer than typical chromatographic peak widths and is possibly distributed over a portion of the column that is long compared to typical terminal band lengths for normal peaks [41,74,91]. These processes are referred to as band broadening in time and band broadening in space, respectively, and are responsible for degradation of column performance and possibly peak distortion, unless an effective focusing method is employed. Band broadening in space is the primary cause of peak distortion in cold on-column injection [40,89,92]. The plug of liquid leaving the syringe flows into the column inlet or retention gap, closing the column bore. This plug is then pushed further into the column or retention gap by the carrier gas until the liquid is spread over the surface of a considerable length of the inlet. During the primary flow of liquid a layer of sample is left behind the plug, reducing the length of the plug until, eventually, the whole sample is spread as a stable film on the column or retention gap wall. This layer of sample is called the flooded zone [93]. Its length depends primarily on the sample volume, whether the solvent wets the column wall or not, and the column temperature. It is amplified by large volume injections, sample solvents incompatible with the properties of the stationary phase or surface of the retention gap, and solutes with an elution temperature more than about 50°C higher than the injection temperature. Cold trapping and solvent effects are the mechanisms used to focus bands broadened in time or space, in some cases facilitated by using a retention gap.

3.5.6.1 Cold trapping

In the absence of a focusing mechanism the slow introduction of sample into the column causes all solute bands to be broadened equally, Figure 3.10. This band broadening in time is minimized by temporarily increasing the retention power of the column

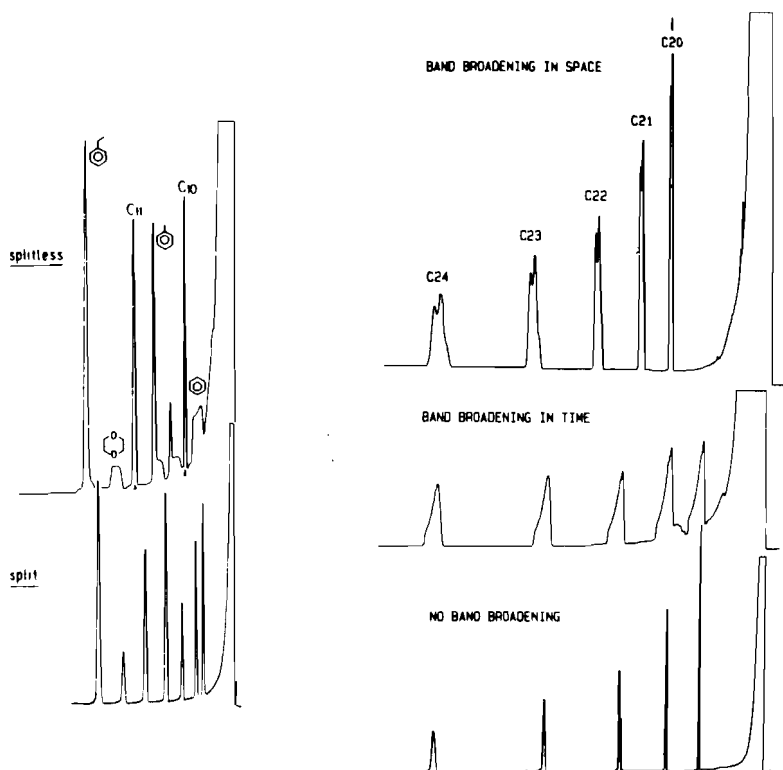


Figure 3.10. Different peak distortion problems due to band broadening in time and band broadening in space observed during hot splitless injection. Band broadening in space is characterized by a broadening which grows proportionally with retention time and may result in peak splitting that is poorly reproducible. Band broadening in time is characterized by a constant broadening of all peaks. Partial solvent trapping results in characteristic chair and stool shaped peaks. (Adapted from ref. [40 and 41]; ©Wiley-VCH).

inlet during sample introduction. To be effective, the migration velocity of the sample entering the column at the start of the injection period must be sufficiently retarded to allow portions of the sample entering at the end of the injection period to catch up with it. This can be achieved by lowering the column temperature, called cold trapping, or by a temporary increase of the retention power of the column inlet with the injection solvent acting as a liquid phase, called solvent effects. Cold trapping is usually performed in the absence of solvent effects by maintaining the column inlet temperature not less than 15°C below the solvent boiling point to minimize condensation of solvent vapors in the column inlet. A minimum temperature difference of about 80°C between the inlet temperature and the elution temperature for the solutes of interest is usually required for efficient cold trapping. The efficiency of cold trapping as a focusing mechanism is determined by the ratio of the migration speed of the solutes at the injection and elution

temperatures. Cold trapping is frequently used when temperature program separations are required.

3.5.6.2 Solvent effects

A prerequisite for achieving band focusing by solvent effects is the formation of a temporary liquid film in the column inlet of sufficient retention power to delay migration of the sample. The solvent should provide strong interactions with the sample to avoid partial solvent trapping, which can lead to distorted peaks similar to those shown in Figure 3.10, as well as wet the column wall, otherwise a stable film will not be formed. For vaporizing injectors the column temperature must be low enough to ensure sufficient solvent is condensed from the solvent saturated carrier gas to form a film that persists until vapor transfer is complete and for on-column injection low enough that the liquid flows from the syringe needle as a plug. In most cases, these conditions will be met if the column temperature is below the solvent boiling point. Solvent trapping is an effective focusing mechanism for volatile solutes with normal elution temperature no more than about 50°C above the column inlet temperature. Solvent trapping only occurs under conditions that allow solvent evaporation in the flooded zone to proceed from the rear to the front. Volatile solutes do not remain where the evaporating solvent deposits them but migrate with the unsaturated carrier gas. If they migrate at least as rapidly as the rear of the sample layer during solvent evaporation, they end up being focused into a sharp band located at the place where the last portion of solvent evaporated. Fully trapped solutes are released from the flooded column inlet during a very short time eliminating measurable band broadening in time. Fully trapped solute bands are also narrow in terms of their spatial distribution if their volatility is not too low. Solute of low volatility are deposited throughout the flooded zone and are subject to band broadening in space.

Full solvent trapping does not influence the separation process in the column. Phase soaking, by contrast, is a solvent effect that takes place in the separation column due to an increase in the retention power of the stationary phase. This increase in retention power results from the swelling of the stationary phase by the temporary sorption of condensed solvent vapors. The increase in volume of the stationary phase can be accompanied by a change in selectivity if the solvent has different retention properties to those of the stationary phase. Solute are affected by phase soaking if they migrate through part of the separation column overlapping with the solvent band, the front and rear of the solute band being retained unequally by the soaked and solvent free stationary phase. Phase soaking tends to sharpen solute peaks eluted in front and close to the solvent peak and is less effective for peaks well removed from the solvent peak. It is only an effective focusing mechanism for peaks eluted at low column temperatures.

3.5.6.3 Retention gaps

Band broadening in space can be largely eliminated by making the injection at a column temperature slightly above the solvent boiling point at the carrier gas inlet pressure. This minimizes the formation of a flooded zone but quantitative aspects of this approach

are not always satisfactory and the peak widths of volatile sample components may be broadened due to the absence of solvent effects as a focusing mechanism. A more universal solution is the use of a retention gap.

Retention gaps are column inlets with a reduced retention power compared to the separation column [40,91,94-97]. They function as guard columns to protect the separation column from contamination by involatile residues; wide bore retention gaps permit the use of autoinjectors with regular syringe needles and standard bore separation columns; and retention gaps facilitate the injection of large sample volumes. Most retention gaps are simply different lengths of uncoated fused silica tubing, deactivated by the same techniques as those used for column preparation, when required, to modify their solvent compatibility or surface activity. As a general guide, typical retention gaps should be about 25-30 cm long for each μl of solvent injected unless concurrent solvent evaporation is used. It is also important that the solvent wet the retention gap surface in order to produce a film of liquid on the column wall. Focusing of solute zones occurs because the retention power of the separation column is 100 to 1000 times greater than that of the retention gap. Solutes migrate through the retention gap at temperatures well below those required to cause elution from the separation column. Thus, they arrive at the entrance to the separation column at a temperature too low for significant migration and accumulate there until a temperature is reached at which migration starts. Retention gaps are essential when sample volumes greater than about 5 μl and up to several hundred μl are injected. They are strongly advised for the routine analysis of dirty samples of any volume to protect the separation column from contamination by involatile matrix components.

3.5.7 Liquid Chromatography-Gas Chromatography

The development of large volume injection techniques for gas chromatography opened the door for subsequent developments in coupled liquid chromatography-gas chromatography (LC-GC) systems [98-106]. Liquid chromatography provides efficient matrix simplification and transfer of defined eluent fractions to the gas chromatograph for high-resolution separation and detection. Direct transfer of eluent fractions to the gas chromatograph reduces the possibility of contamination or losses during solvent removal improving sample detection limits and repeatability. Compared with conventional sample preparation procedures the greater resolving power of modern liquid chromatography together with the possibility of on-line monitoring for fraction selection provides cleaner and well-defined extracts for separation by gas chromatography. For volatile analytes gas chromatography offers unsurpassed separation performance and a wide choice of reliable detection options. Through automation of the total process unit costs are reduced and laboratory productivity increased. While most practical applications are limited to normal phase and size-exclusion liquid chromatography using volatile organic solvents progress has been made in the more difficult problem of suitable methods for transferring aqueous solution from reversed-phase liquid chromatography (section 3.5.8). LC-GC lacks a general candidacy

and major applications tend to reflect the interests of the small number of research groups that have contributed to its development. Typical examples are applications in food quality control (e.g. mineral oil contamination, steroids and minor components in oils and fats, toxic contaminants, etc.) and detailed analysis of petrochemical products and essential oils [98,101,104-109].

Separations by liquid chromatography occur with concurrent dilution requiring the transfer of relatively large volume fractions to the gas chromatograph. Early solutions to this problem used either narrow bore liquid chromatography columns (< 1 mm) operated at low flow rates (< 70 $\mu\text{l}/\text{min}$) or wider diameter liquid chromatography columns and a split interface that directed only a portion of the eluent fraction to the gas chromatograph to minimize transfer volumes. Neither approach maximizes the possibilities of LC-GC, particularly for trace analysis. Most contemporary applications use either the loop interface with concurrent eluent evaporation or the on-column interface with a retention gap and partial concurrent eluent evaporation. With these techniques transfer fractions of 50 μl to several ml are easily handled allowing a nearly unrestricted choice of column diameter and sample size.

For fractions transferred to the gas chromatograph with full concurrent eluent evaporation the components of interest are isolated in the loop of a switching valve, Figure 3.11 [98-102]. Opening the valve allows the contents of the loop to be displaced into the thermostatted retention gap driven by the carrier gas. At a temperature above the pressure corrected solvent boiling point the penetration of the eluent plug into the retention gap is quickly arrested by the vapor pressure generated by solvent evaporating from the front of the plug. The length of the flooded zone is generally less than 1 m and short retention gaps of 1 – 2 m are adequate for the introduction of eluent volumes of any reasonable size. Since the column carrier gas flow starts only after the evaporation step is complete an early solvent vapor exit, located before the separation column, is a useful addition to accelerate the discharge of solvent vapors and to avoid the passage of large amounts of vapor through the column and detector. Solvent trapping and phase soaking are inefficient band focusing mechanisms under the conditions of solvent evaporation employed. Cold trapping must be relied upon for this purpose. Consequently, sharp peaks will be obtained only for solutes with an elution temperature at least 40 - 120°C higher than the transfer temperature. The method is limited, therefore, to the analysis of solutes of moderate to low volatility. It is reasonably straightforward to obtain eluent introduction rates of 80-150 $\mu\text{l}/\text{min}$ providing acceptable transfer times for eluent fractions up to several ml.

Retention gap techniques with on-column interfaces are used to analyze those analytes that are poorly retained during full concurrent eluent evaporation [98-102,105]. The eluent fraction is pushed by the liquid chromatography pump into the thermostatted retention gap where it is mixed with carrier gas and evaporated at a temperature below the pressure corrected eluent boiling point. A layer of condensed solvent (flooded zone) is formed in front of the evaporation site that acts as a temporary retaining liquid phase. Partial concurrent eluent evaporation during transfer allows the use of shorter retention gaps and an increase in the eluent volume that can be transferred. A 10 m x 0.53 mm

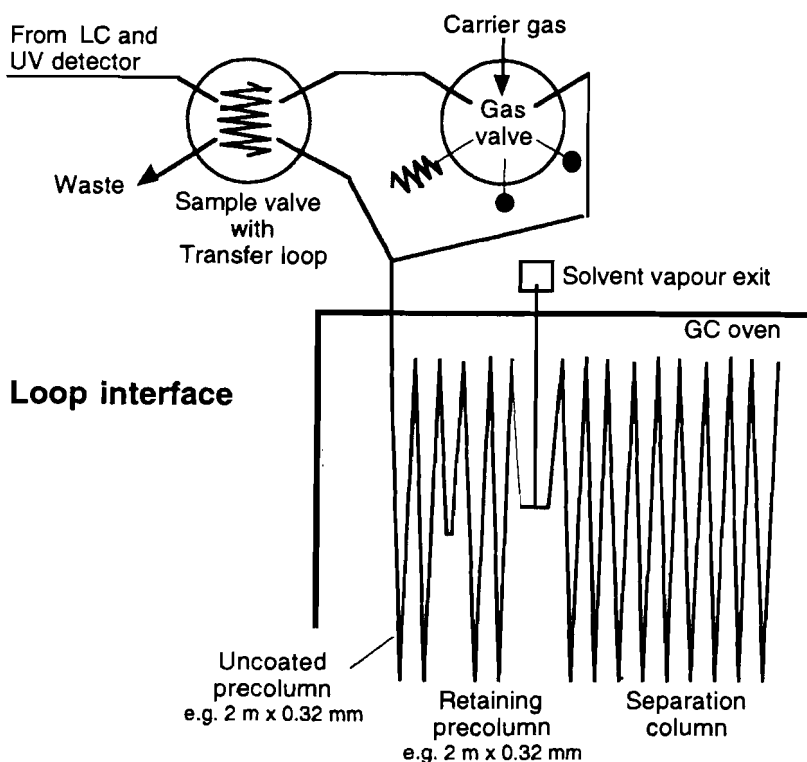


Figure 3.11. Schematic diagram of a loop type interface with concurrent eluent evaporation for LC-GC. (From ref. [100]; ©Elsevier).

I. D. retention gap has the capacity to retain up to about 250 μl of eluent extended to about 1.0 ml with 75% partial concurrent eluent evaporation. An early solvent vapor exit and high gas flow rates allow increased evaporation speeds and faster eluent transfer. A retaining precolumn before the solvent vapor exit or restriction in the vapor outlet is required to prevent loss of volatile analytes by slight delay in timing the closure of the vapor exit. Optimization of the transfer conditions depends in a complex way on the interdependent parameters of the retention gap length (controls the length of the flooded zone), the eluent transfer rate, the rate of solvent evaporation, and timing of the closure of the vapor exit. Retention gap techniques can yield sharp peaks for analytes with elution temperatures at least equal to the column oven temperature during transfer.

3.5.8 Injection or Transfer of Aqueous Solutions

The introduction of aqueous solutions into a gas chromatograph equipped with an open tubular column is far from a straightforward process. Water is a poor solvent for promoting the focusing mechanisms required for successful sample introduction and

aggressively destroys the deactivation layers used to minimize adsorptive properties of columns and inlets [69,70,100-102, 110,111]. Water does not form stable films on deactivated glass surfaces or surfaces coated with common stationary phases. In the absence of a flooded zone the only available focusing mechanism is cold trapping. The relatively high temperatures required for the efficient vaporization of water restricts the effectiveness of cold trapping to solutes of low volatility. In addition, the large volume of vapor produced leads to excessive band broadening and is incompatible with the operation of several common gas chromatographic detectors. The process of evaporating liquid water from hot surfaces (more so than contact with steam) destroys silane-deactivated surfaces and even poly(siloxane) phases. To avoid these problems a number of common sample preparation procedures that isolate and transfer the analytes to a more favorable solvent or the vapor phase are generally used to analyze aqueous solution (e.g. liquid extraction, solid-phase extraction, headspace methods, etc.) [77,104,112,113].

Cold on-column injection techniques are feasible for aqueous solutions if the sample solvent contains less water than can be azeotropically evaporated from the flooded zone (e.g. 28 % water in 1-propanol) but durability of deactivated surfaces is a problem. Hot splitless injection techniques are limited to small volumes by the large volume of vapor produced during injection and to solutes of low volatility for cold trapping to be an effective band focusing mechanism. The most promising approaches use a PTV injector operated in the solvent split or splitless mode with a packed liner containing Tenax and/or Carbofrit [69,70,102,105,114,115]. With controlled injection flow rates of 10 – 70 $\mu\text{l}/\text{min}$ (the slow introduction rate is to accommodate the strong cooling of the chamber by the evaporating water) several hundred microliters of water can be injected. Volatile compounds are lost by gas discharge with the evaporating water and compounds of low volatility by excessive retention in the liner at the desorption stage. Separating the solvent evaporation and solute/solvent separation stages using two PTV injectors and a retention cascade of successively increasing retention power offers a more comprehensive approach suitable for injection or transfer in LC-GC of aqueous solutions [100,111]. The aqueous sample is evaporated at a controlled rate in the first PTV injector. The vapor outlet of this injector is connected to the second PTV injector by a retaining precolumn (a short length of a coated column) thermostatted by the column oven. During sample introduction the second PTV injector is maintained at a low temperature just sufficient to avoid condensation of water vapor. It contains an injection liner packed with Tenax and Carbotrap sorbents in series to trap volatile compounds. For desorption the carrier gas flow is reversed, the second PTV injector heated rapidly to its operating temperature, and the trapped analytes returned through regions of decreasing retention power to the separation column. Aqueous solutions (including water) can be injected at about 100 $\mu\text{l}/\text{min}$ with the retention cascade providing improved trapping efficiency for volatile compounds. At present no single method is suitable for all sample types, and no suitable methods exist for the introduction or transfer of some sample types. The methods discussed in this section represent a promising beginning on the road to a permanent solution.

Indirect methods of handling aqueous solutions involve phase switching [69,77,104-106]. The analytes are isolated from the aqueous solution and transferred to a volatile organic solvent for introduction into the gas chromatograph. The perfection of large volume injection techniques for volatile organic solvents made these techniques attractive. The isolation step is performed by continuous or in-vial microextraction liquid-liquid extraction (LLE) or solid-phase extraction (SPE). These techniques are important sample preparation methods in their own right and are facilitated by convenient automation options. On-line coupling of SPE-GC or LLE-GC allows better sample utilization or a reduction in the size of the sample required for analysis compared with classical off-line methods and the use of a closed system minimizes the possibility of contamination from external sources.

In on-line SPE-GC the analytes are isolated from aqueous samples on a hydrophobic precolumn containing either a macroporous polymer or siloxane-bonded silica sorbent of a medium particle size (10-40 μm) or a number of thin particle-loaded membrane disks assembled to form a continuous bed. The precolumns are typically about 2 - 20 mm long with an internal diameter of 1 - 4.6 mm and are mounted on a conventional 6- or 10-port switching valve (see Figure 3.9) [116-118]. The sample processing part of the interface must provide for automation of the standard operating procedures of SPE: (i) sorbent conditioning with organic solvent (e.g. 100 μl) followed by water (e.g. 2.5 ml); (ii) sample application (e.g. 10 ml at 1-10 ml/min); (iii) water rinse to remove salts and polar compounds (e.g. 1.9 ml); (iv) drying of the cartridge (e.g. nitrogen gas at 70 ml/min for 30 min); and (v) desorption of the analytes with a small volume of solvent such as methyl or ethyl acetate (e.g. 50-100 μl). The desorption solvent is transferred to the gas chromatograph via a narrow bore deactivated fused silica capillary to an on-column injector with a retention gap and possibly retaining precolumn and an early solvent vapor exit. Transfer occurs under partial concurrent solvent evaporation conditions with the solvent vapor exit initially open during transfer and closed just before completion of the evaporation process. It is very important that the drying process is efficient since even a few percent of water absorbed by the desorption solvent will cause destruction of the deactivated retention gap. Apart from purging with nitrogen at room temperature, which is a slow process, a drying cartridge containing anhydrous sodium sulfate or silica gel can be inserted between the SPE cartridge and the inlet of the gas chromatograph [118].

The recovery of volatile analytes by partial concurrent solvent evaporation was significantly improved by using a volatile desorption solvent (e.g. methyl acetate). This allowed a low transfer temperature to be used and the addition of a presolvent to ensure the early formation of a solvent film to enhance solvent trapping [77,117]. The concentration of analytes in the initial portion of the desorption solvent is high and falls to a low level or zero at the completion of the desorption process. The early escape of weakly trapped volatile analytes deposited at the front of the solvent film, where the analyte concentration is highest, is the main cause of their low recovery. Volatile analytes poorly trapped by the solvent film in the retention gap are transported through the gas phase faster than the front of the solvent film advances during solvent transfer.

Once these analytes have moved ahead of the film boundary they are no longer retarded and are lost through the solvent vapor exit. The loss of partially trapped analytes is more critical in on-line SPE-GC than for conventional large volume injection because of the nonuniform distribution of analytes in the solvent film. The introduction of a small volume of organic solvent, presolvent, immediately in front of the sample plug to ensure that a solvent film is already present in the retention gap when sample transfer starts, can be used to minimize the loss of volatile analytes. Acceptable recovery of analytes as volatile as xylene and chlorobenzene was obtained using an on-column interface when 30-50 μl of methyl acetate was introduced immediately in front of 50 μl of the same desorption solvent.

Loop-type interfaces with full concurrent solvent evaporation are more robust and easier to automate than the on-column interface with partial concurrent solvent evaporation but are restricted to the analysis of solutes of low volatility. A significant advance in this regard was made by the addition of cosolvent trapping [119] or partial concurrent solvent evaporation in a long retention gap capable of holding at least half the transfer volume as liquid [77,120,121]. The latter interface is relatively easy to optimize and operate, while its application range closely overlaps that of the on-column interface. The interface is equipped with a 6-port and a 14-port switching valve. The 6-port valve is used to divert the carrier gas either to the gas chromatograph or to the 14-port valve, on which the SPE cartridge and two loops for storing organic solvent are mounted. The first loop contains the presolvent and is usually of smaller volume (e.g. 50 μl). The second loop contains the solvent used to desorb the analytes from the SPE cartridge and to transfer them to the gas chromatograph. After the sample processing steps are completed and the cartridge dried, both valves are switched simultaneously and the solvent vapor exit opened. The carrier gas pushes the contents of both loops, the second loop via the SPE cartridge, into the retention gap. Both valves are switched to the load position when a preselected pressure drop corresponding to near completion of the evaporation process is recorded and the solvent vapor exit closed after a selected delay time. The transfer is carried out at a temperature just above the solvent boiling point. The use of relatively small desorption solvent volumes (50-100 μl) allows transfer temperatures below those used for full concurrent solvent evaporation without the need for very long retention gaps. When this approach is combined with the use of a presolvent that swells the stationary phase of the retaining precolumn, analytes as volatile as undecane can be transferred successfully with ethyl acetate as the desorption solvent and presolvent.

Thermal desorption from the SPE cartridge is a further possibility [77,122]. In this approach, the sample is introduced at a controlled speed into the packed liner of a PTV injector set to a low temperature with the water eliminated via the split vent. Salts and involatile polar material are rinsed from the sorbent with water and the sorbent dried by purging with a high carrier gas flow rate. The trapped analytes are subsequently desorbed in the splitless mode by rapidly heating the PTV to the injection temperature. The most commonly used sorbents are Tenax and Carbofrit. The method is restricted to a narrow range of applications by the low breakthrough volume of polar analytes on the

small sorbent bed (< 10 mg), typically 0.1 to 1.0 ml, and by the thermal stability and excessive retention of analytes of low volatility at the desorption stage.

3.6 SUPERCRITICAL FLUID INLETS

The main requirement of an interface for coupling supercritical fluid extraction (SFE) or chromatography (SFC) to a gas chromatograph is to provide an efficient mechanism to collect and focus the analytes introduced over a long time at high gas flow rates generated from the depressurization of the supercritical fluid. This can be achieved using standard split/splitless, on-column or PTV inlets for gas chromatography with only minor modification [123-128]. For heartcutting fractions from a supercritical fluid chromatograph a switching valve and T-piece providing a split flow to a detector for monitoring fractions was used to define the regions of the chromatogram to be switched to the gas chromatograph [127]. Most studies, however, involve the on-line coupling of supercritical fluid extraction to gas chromatography. In this case, the transfer line from the extraction cell is also the restrictor used to control the flow of fluid and maintain supercritical fluid conditions to the point where decompression occurs in the inlet of the gas chromatograph. A crimped stainless steel capillary (0.25 mm I.D.) or a length of fused-silica capillary tube (10-50 μ m I.D.) provide suitable transfer lines.

Most studies report results for pure carbon dioxide or carbon dioxide with a small amount of organic solvent modifier. Accumulation in a sorbent trap or sorbent packed liner of a PTV injector is probably the best approach for handling fluids containing significant amounts of organic solvent [125,126]. In the latter case extracted components are initially collected in a retaining liner with elimination of carbon dioxide and solvent vapors through the split vent. The trapped components are then transferred to the column in the splitless mode by rapidly heating the injector to its operating temperature. The critical parameter is the temperature of the injector during trapping, which should be higher than the boiling point of the solvent modifier to avoid displacement of the analytes from the retaining liner by condensed solvent.

The conditions used for extraction have a lot to say about the selection and optimization of the interface for on-line SFE-GC. Transfer times are typically 15 – 60 min with a fluid flow that generates about 50 – 1000 ml/min of gas after depressurization. Small extraction cells (0.1 – 1.0 ml) are generally preferred for on-line coupling. These cells can be operated at optimized flow rates compatible with most interfaces and provide sufficient extract for analysis in most cases. The main limitations on the smallest sample size that can be analyzed are the concentration of the target analytes and whether a representative sub-sample can be obtained. At the other end of the sample scale up to about 1 g can be handled with an on-column interface and about 15 g with a split interface based on their ability to operate at typical fluid flow rates with extractors of different size.

In the split mode the supercritical fluid is depressurized at the tip of the transfer capillary located inside the liner of the heated inlet. The analytes are continuously

vaporized in the liner and homogeneously mixed with carrier gas and carbon dioxide over the time required for dynamic extraction, with most of the gases exiting via the split vent and a small amount entering the column inlet. Discrimination-free split operation is obtained at sufficiently high temperatures, even when significant amounts of organic modifier are present, provided that the analytes are reasonably soluble in the modifier. Sample focusing at the column inlet relies on cold trapping by the stationary phase. In order to obtain good peak shapes a low column inlet temperature, thick film columns and short dynamic extraction times are preferred. Cryotrapping may be necessary to achieve sharp chromatographic peaks for the most volatile compounds. Temperatures $> -50^{\circ}\text{C}$ are employed due to the poor partitioning properties of many immobilized phases at lower temperatures and increasing problems with blocking of the column inlet with frozen water and carbon dioxide. All but the most volatile compounds, however, can be trapped at room temperature with stationary phase film thickness appropriately adjusted to accommodate efficient trapping while at the same time providing acceptable elution temperatures for the separation. The split interface is reasonably tolerant of coextracted water and involatile material that remains in the injection linear and/or retention gap. Except for trace analysis the fact that only a fraction (0.1 – 10 %) of the extracted analytes is transferred to the column is not a major problem and the simplicity and ruggedness of the split interface has resulted in it becoming the most widely used.

When on-column injection is used the end of the transfer capillary is inserted into the column inlet or retention gap where decompression of the supercritical fluid occurs. Carbon dioxide gas exits through the column and the seal made between the restrictor and septum (unless a closed injector is used). The analytes are focused by cold trapping in the stationary phase. The transfer line must be physically removed from the injector at the completion of the extraction to establish the normal carrier gas flow for the separation. Analyte transfer to the column is virtually quantitative but blockage of the restrictor is more common and involatile material accumulates in the injection zone eventually degrading chromatographic performance. The on-column interface is probably a better choice for trace analysis of relatively clean extracts with modest fluid flow rates than the split interface. When optimized both the on-column and split interfaces provide essentially identical peak shapes to those obtained using conventional solution injection.

3.7 VAPOR SAMPLE INLETS

A number of important sample preparation techniques rely upon gas extraction or the analysis of samples in the gas phase. These samples usually contain low concentrations of volatile analytes and higher concentrations of water vapor in a low molecular weight gas or air. Direct sample introduction by syringe or valves is only suitable for small volumes of relatively concentrated samples (section 3.4.2). More common sample introduction methods involve analyte accumulation by sorbent or cryogenic trapping followed by vaporization in the presence of a flow of gas to transport the

analytes to the column. These methods are further examples of solventless injection and rely entirely upon cold trapping by the stationary phase for band focusing. Important automated sample introduction methods based on sorbent trapping include static and dynamic headspace (purge-and-trap) and solid-phase microextraction. Condensation in a cryogenic trap followed by rapid heating is an alternative method of accumulation and sample introduction to thermal desorption as well as a suitable inlet for fast gas chromatography and coupled-column gas chromatography.

3.7.1 Thermal Desorption

Typical sorbents used for analyte accumulation are porous polymers (e.g. Tenax), graphitized carbon blacks and carbon molecular sieves, and in special cases inorganic oxides (e.g. silica gel). These are typically packed into stainless steel tubes 89 mm x 5 mm I. D. (volume \approx 3 ml containing 0.2 – 1.0 g of sorbent) or glass tubes 89 mm x 4 mm I. D. (volume \approx 2 ml). The outer diameter of the tubes is 6 mm. The sorbent bed occupies the central portion of the tube up to a maximum of 60 mm and is held in place by stainless steel gauze or glass wool plugs [129-132]. These standardized dimensions assure compatibility with automated thermal desorption devices and are suitable for trapping a wide range of volatile compounds in atmospheric and dynamic headspace methods. For sampling mixtures of a wide volatility and polarity range segmented beds of different sorbents are used. Samples are drawn through the tubes, usually at ambient temperature, by a pump in atmospheric sampling or by pressure in a closed system, such as purge and trap.

The trapped analytes are transferred to the column by heating the sampling tube to a high temperature while purge gas flows in the opposite direction to the sample flow employed for trapping. The desorption temperature is usually set just below the boiling point of labile compounds or at the highest temperature allowed by the sorbent for thermally stable compounds (\approx 300°C for carbon and Tenax) with a purge gas flow of 30 – 50 ml/min through the sampling tube. The slow desorption kinetics and high purge gas flow rates are compatible with packed columns allowing direct transfer with cold trapping by the stationary phase providing band focusing. For open tubular columns a two stage desorption process, sometimes aided by a stream splitter, is generally required. The second accumulation trap is either a small-scale sorbent trap or cryogenic trap (section 3.7.2). Prior to thermal desorption the sampling tube is purged with carrier gas to displace air from the sorbent bed to avoid artifact formation by high temperature reaction of the sorbent or analytes with oxygen. The sampling tube is then thermally desorbed at the chosen temperature using resistive heating or cartridge heaters housed in a heating block around the sampling tube for the time required to complete the desorption process (e.g. 5 – 15 min). Alternatively, carbon adsorbents can be housed in ceramic sampling tubes and thermally desorbed by direct microwave heating of the adsorbent [39,133]. The analytes are accumulated in a second small-scale sorbent trap or cryogenic trap after splitting the flow of gas from the sampling tube to adjust the sample concentration to the capacity of the open tubular column, if required. The

small-scale sorbent trap contains less than 100 mg of sorbent and has a low thermal mass allowing rapid heating ($> 35^{\circ}\text{C/s}$) to minimize band broadening of the transferred analytes [134]. The small-scale sorbent trap usually contains Tenax, a dual carbon sorbent bed, or the same sorbent used for the sampling tube. For accumulation the small-scale trap is maintained at a temperature close to room temperature or cooled to -30°C by a solid state thermoelectric Peltier cooler [135]. A suitable combination of sorbent and temperature allows compounds as volatile as ethane to be successfully trapped. Desorption from the small-scale trap occurs at a slightly higher temperature than was used for desorption of the sampling tube with a flow rate of about 3 ml/min and desorption time of 1 - 5 min. A split flow between the trap and the column can be used to further adjust the sample concentration to the sample capacity of the column. Accumulation of water during the sampling stage can cause problems in the transfer process by ice formation in the column inlet if subambient temperature trapping is used and by distorting the peak shape of separated compounds. The selection of sorbents with a low water retention for sampling [136,137], the use of a dry purge step prior to desorption, or various desiccant and condensation techniques (section 3.7.3) are used to manage the water problem. When cryogenic trapping is used as the intermediate accumulation trap its efficiency depends primarily on its temperature, coating or adsorbent packing (if used) and gas flow rate [138]. The chromatographic performance of the injected sample depends on the heating rate of the trap, the carrier gas flow rate and the efficiency of cold trapping by the stationary phase as a focusing mechanism. Fully automated thermal desorption analyzers using a sequential tube sampler are available. Thermal desorption devices are usually designed as accessories to interface to standard gas chromatographs with minimal modification. A thermostatted short length of deactivated fused silica or silica-lined stainless steel capillary tube or a syringe needle attached to the sampling tube (short path thermal desorption) acts as a transfer line terminated in a standard hot split/splitless injector.

The standard apparatus used for thermal desorption is equally suitable for thermal extraction. Thermal extraction is used for the analysis of volatile compounds in solid samples of low moisture content (e.g. plant materials, soil, polymers, etc). In this case, a few milligrams of sample in place of the sorbent contained in a standard desorption tube is heated to a temperature below its melting or decomposition temperature. The volatile compounds released from the sample are accumulated and transferred to the column in the same way as for thermal desorption.

Solid-phase microextraction employs a polymer-coated, fused-silica fiber housed in a modified syringe as a sampling device, Figure 3.12 [139,140]. The holder is equipped with an adjustable depth gauge for precise positioning of the fiber in the sample and gas chromatographic inlet. The fiber is immersed in liquid samples or suspended in the headspace above liquid or solid samples for sorption of the analytes. The fiber is then retracted into the protective needle shroud and introduced into a hot split/splitless inlet for transfer of the analytes to the column by thermal desorption. The standard injection liner is replaced by a narrow bore liner (0.8 mm I. D.) to facilitate sharp injection bands by generating a high velocity of hot gas around the fiber coating. Typically, the

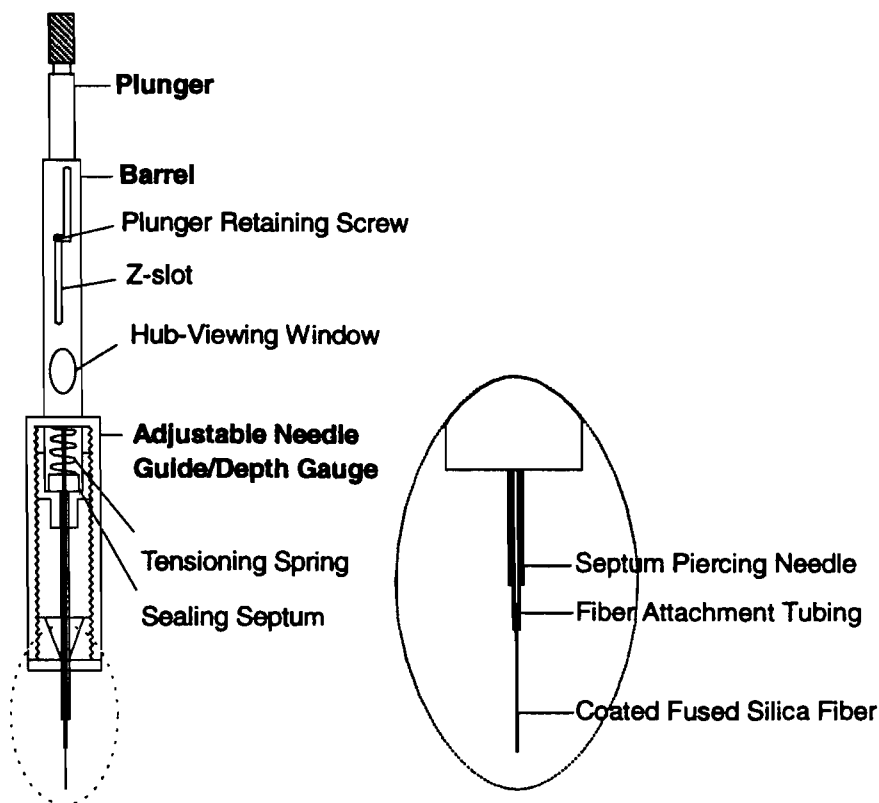


Figure 3.12. Schematic diagram of the sample handling device for solid-phase microextraction. (From ref. [139]; ©Wiley-VCH).

injector is set at the maximum temperature tolerated by the fiber coating for thermal desorption and the desorption time optimized empirically to obtain quantitative transfer. Cold trapping by the stationary phase is responsible for band focusing and is generally optimized by careful selection of the stationary phase film thickness and the initial column temperature. Cryogenic trapping is rarely required if a narrow bore liner is used. Strong interactions between polar analytes and silica of the fiber core can result in poor desorption efficiency for thin-film, coated fibers [140].

3.7.2 Cryogenic Traps

Low temperatures are used in two ways to trap volatile organic compounds from the gas phase [141,142]. Volatile compounds can be retained by condensation if the temperature of the trap is below the dew point for the compound. For low concentrations of volatile compounds very low temperatures (e.g. liquid nitrogen) are required. The trap itself can

be an empty tube, a length of coated capillary column or a packed tube containing a nonretaining material (e.g. glass beads) [138,143-147]. The nonretaining packing provides an increase in the surface area of the trap for collision with vapor molecules while allowing rapid thermal desorption for their transfer to the column. Retaining materials may improve the trapping efficiency of very volatile compounds but limit the desorption rate and efficiency of less volatile compounds [145]. Slow desorption results in poor chromatography in the absence of an effective column focusing mechanism and irreversible adsorption in poor quantitative accuracy. Cryogenic focusing refers to cold trapping by the stationary phase (or retaining material) at temperatures below ambient. In this case the trap temperature need only be low enough to effectively retard the migration of the solute by the stationary phase. The temperature of the trap must be above the glass transition temperature of the stationary phase since the retention power of solid phases is usually no greater than that of uncoated traps [148].

Cold traps are usually simple and based on a tube within a tube design. The inner tube is a length of metal or fused silica capillary surrounded by a wider metal or polymer tube through which cold gas is circulated around the annular space between tubes. The cold trap is usually located in the oven of the gas chromatograph connected to the base of the injector. In most cases the fused silica capillary inner tube is simply a portion of the column inlet or retention gap. The cold gas is obtained by passing gas through a tube contained in a cryostat or generated from the expanding vapors above liquid nitrogen or liquid carbon dioxide. The lowest temperatures that can be obtained are -196 to -180°C for liquid nitrogen and -65 to -60°C with liquid carbon dioxide. The low thermal mass of the fused silica capillary allows rapid convective heating by the incoming carrier gas, the circulating air in the chromatographic oven, by circulating hot air from an external source, or by resistive heating of the outer tube surrounding the capillary. The selection of the heating method is partly dependent on how trap operation is to be automated as well as the final temperature the trap is to be heated to. A feedback mechanism is usually available to control the flow of cryogen or hot air to allow accurate setting and cycling between set point temperatures with total automation. Cryogenic traps are generally more efficient and cost effective than repeatedly cooling and heating the whole column oven. For occasional use a Dewar flask containing liquid nitrogen into which the first meter or so of the separation column or a metal precolumn is immersed is quite effective [149]. Subsequently the Dewar flask is removed and the column oven rapidly heated to the initial temperature for the separation.

Cryogenic traps are a convenient accumulation and injection device for fast gas chromatography and are also used in coupled-column gas chromatography, where a heartcut sample is collected and focussed from the first column, and reinjected into the second column. The main requirement for a cryogenic trap used in these applications is efficient accumulation over time with rapid injection of the collected analytes as a narrow pulse in both time and space. For fast gas chromatography the sample is condensed in a short metal capillary tube using circulated cold gas and then rapidly vaporized (10 – 20 milliseconds) by ballistically heating the tube with a capacitance discharge [150-152]. For long traps (> 20 cm) the gas flow direction through the trap

is reversed for injection resulting in narrower bandwidths and reduced memory effects. Potential thermal decomposition problems are also minimized because of the reduced sample residence time in the hot metal tube. For shorter traps (< 10 cm) flow reversal is less useful. Electronic pressure control allows low flows to be used for trapping and high flows during thermal desorption to obtain the same effect as flow reversal. Commercially available systems provide initial bandwidths of 5-20 milliseconds.

3.7.3 Headspace Analyzers

Headspace gas chromatography is applied to a variety of extraction techniques employing transfer of volatile compounds to a gas phase for subsequent analysis by gas chromatography [142,125-128]. If the sample is in thermodynamic equilibrium with the gas phase in a closed vessel, then this method of analysis is referred to as static headspace. If a carrier gas is passed over the sample or through the sample in the form of a stream of small bubbles and the extracted volatile compounds accumulated in a cryogenic or sorbent trap, then the method is generally referred to as dynamic headspace, purge-and-trap, spray-and-trap [157,158], gas phase stripping, or gas phase sparging. The headspace sampling methods are used primarily for the trace analysis of volatile compounds in samples that are difficult to analyze by conventional techniques. They offer economy of effort and an extract relatively free of the chromatographic problems associated with the sample matrix. On the other hand, the headspace techniques result in a sample that is more or less a diluted gas sample. Its analysis by gas chromatography requires a method that can introduce a large sample volume to obtain the required quantification limits and as fast as possible to avoid band broadening due to delayed sample introduction.

3.7.3.1 Static Headspace Analyzers

The sample, either solid or liquid, is placed in a glass vial of appropriate size and closed with a Teflon-lined silicone septum. The vial is carefully thermostatted until equilibrium is established. The gas phase is then sampled by syringe for manual procedures (section 3.4.1) or automatically using one of a large number of commercially available pneumatic headspace analyzers [39,142,153,156,159-163]. Pneumatic sampling ensures that both the pressure and volume of the headspace sampled are identical for all samples and standards. A constant pressure is obtained by pressurizing the headspace vials with an inert gas to a pressure at least equal to the column inlet pressure. The sample is then either expanded directly into the column or to a sample loop of a thermostatted gas-sampling valve. Typical headspace sample volumes tend to be 0.5-3.0 ml, which can be quickly transferred to and easily handled by packed columns. For open tubular columns with flow rates of 1-2 ml/min complete sample transfer is too slow to provide sharp injection bands. Flow splitting allows the rapid transfer of the sample to the column without discrimination but sensitivity is compromised as only a portion of the sample reaches the column. The alternative approach is to transfer the whole sample to the column, which is then focused at the column inlet by cryogenic trapping.

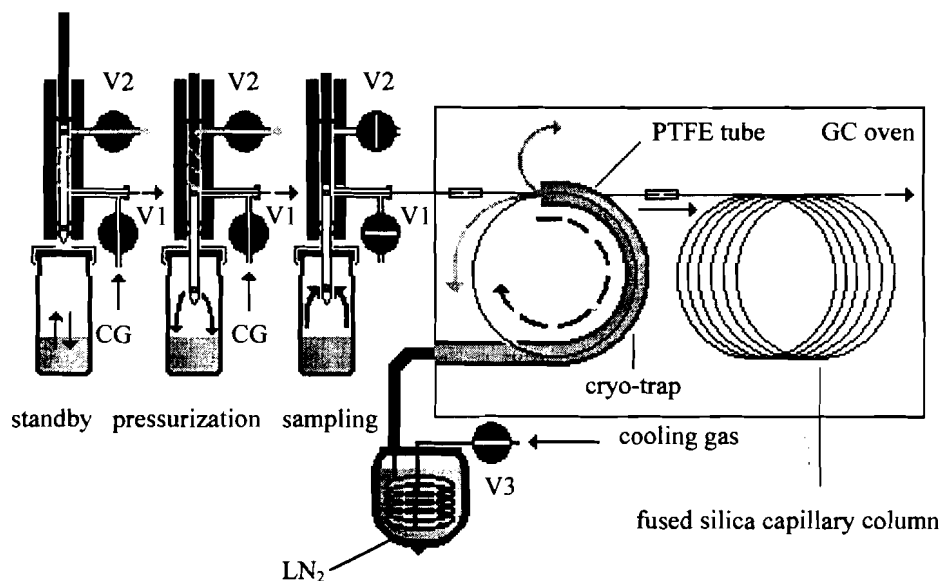


Figure 3.13. Schematic diagram of the balanced pressure sampling system for automated splitless static headspace gas chromatography with cryogenic trapping. V_1 = solenoid valve in the carrier gas (CG) line; V_2 = solenoid valve for the purge gas; and V_3 solenoid valve for the cooling gas. (From ref. [142]; ©Elsevier).

Operation of the pressure/loop interface (gas sampling valve) is straightforward [156,160]. The gas flow to the headspace sampler is split into two flow paths: one path is flow controlled and provides a constant flow of carrier gas which passes from the headspace sampler through the heated transfer line to the gas chromatograph; the second flow path is pressure regulated and, in the standby mode, the sample loop and sampling needle are flushed continuously by the gas flow. After the sample has equilibrated, the sampling needle pierces the septum and the flow of gas pressurizes the headspace vial to the desired pressure. The headspace gas is then allowed to vent through the sample loop to atmosphere via a backpressure regulator. The headspace gas filling the loop has a volume defined by the loop volume, a temperature determined by the thermostat and is at a pressure set by the backpressure regulator (usually the column inlet pressure). Once filled, the sample loop is placed in series with the column carrier gas flow and its contents are driven through the heated transfer line to the sample inlet of the gas chromatograph. The gas-sampling valve, transfer lines and the sampling needle are all thermostatted to prevent sample condensation.

The balanced pressure sampling system, Figure 3.13, comprises a movable needle with two vents and a solenoid valve V_1 in the carrier gas line. The heated needle, which has a hollow part permitting flow in either direction, moves in a heated cylinder and is sealed by three O-rings. In the standby position the lower needle vent is placed

between the two lower O-rings and is sealed from the atmosphere, while the carrier gas flows through valve V_1 to the column. A small cross flow purges the needle cylinder to vent any residual sample vapors through solenoid valve V_2 . At the end of the selected equilibration time the sampling needle descends, pierces the septum cap, and part of the carrier gas flows into the vial raising its internal pressure to the carrier gas inlet pressure. When pressurization is complete valves V_1 and V_2 are closed, disconnecting the carrier gas supply to the column. The pressurized gas in the headspace vial then expands through the sampling needle into the heated transfer capillary connecting the sampler to the gas chromatograph inlet, transferring an aliquot of the gas to the column. At the end of the selected sampling time both valves open again and the carrier gas flow to the column is resumed. The selected pressure and transfer time determine the aliquot of headspace gas introduced into the column. The addition of an auxiliary gas supply line, at a higher pressure than the column carrier gas, and two additional valves, which are placed before valve V_1 , enables the headspace sampler to operate with headspace pressures higher than the column inlet pressure. The increased pressure-sampling mode is convenient for transfer to a split inlet for open tubular columns and for use with packed columns. The balanced pressure sampling technique is convenient for on-column injection using open tubular columns. For on-column injection the transfer capillary is connected directly to the separation column at the bottom of the hot split/splitless inlet.

Cryogenic focusing is used in combination with automated static headspace for focusing the starting bands of the more volatile compounds. The system shown in Figure 3.13 uses a PTFE tube, which jackets the first 20-60 cm of the column. A flow of cold nitrogen gas in the reverse direction to the column carrier gas flow is used to generate a temperature gradient from about -30°C at the column inlet to about -196°C at the coolant inlet. During sample introduction volatile sample components partition into the stationary phase and migrate slowly towards the colder region of the trap until their migration is arrested. When sample transfer is complete the coolant is switched off and the sample vaporized as a narrow band by the heat adsorbed from the column oven and the warm carrier gas entering the column.

3.7.3.2 Purge-and-Trap Analyzers

Purge-and-trap sampling employs a continuous flow of gas through a liquid (usually water) or a homogeneous suspension, with subsequent trapping of the volatile components by a sorbent or cryogenic trap [142,155-158,164-168]. It is used to determine volatile compounds that fail to provide reliable results by static headspace techniques because of unfavorable partition coefficients or because their concentration is too low. The two main variables that affect the efficiency of gas purging are the total extraction volume (product of the purge gas flow rate and purge time) and temperature. Only if the analyte has low water solubility ($< 2\%$ w/w) and is relatively volatile (b. p. $< 200^{\circ}\text{C}$) can quantitative extraction be expected, although at the extreme property range the extraction volume will be large. A major use is

the determination of volatile organic compounds at parts-per-trillion concentrations in surface and wastewater samples for regulatory compliance.

Typical sample sizes for purge-and-trap analysis are 5 ml with a 25-ml vessel available to achieve the low detection limits specified for some analytes. The sample is purged with helium or nitrogen at a specified flow rate, time and temperature. For a broad range of analytes a purge volume of 200-600 ml at a flow rate of about 40 ml/min is sufficient. Most extractions are carried out at room temperature for water or 40°C for samples of solid waste, sludge, soils, etc. The gas extraction efficiency increases with higher temperatures but so does the transfer of water vapor to the trap. Higher temperature extractions require the use of additional techniques for water management, discussed later. The purge gas is introduced below the liquid level through a fine syringe needle or fritted orifice; the finely dispersed bubbles provide maximum surface contact between the gas and liquid phase. Rapid stirring of suspensions also increases the extraction efficiency. The purge vessel has baffled walls or a frit in the purge gas exit line to diminish sample carryover due to foaming, often the most unwelcome of the practical difficulties that might be encountered. The purged volatile organic compounds are usually accumulated on a Tenax trap. For substances with boiling points below 30°C a segmented sorbent trap containing Tenax and carbon molecular sieves is generally used (this is now more popular than the earlier recommended sorbent combination of Tenax, silica gel and charcoal due to lower water retention). The analytes are recovered from the sorbent trap by thermal desorption (section 3.7.1).

Cryogenic trapping is also used with the purge-and-trap technique for the accumulation of volatile organic when open tubular columns are used for the separation, Figure 3.14 [142,169,170]. Cryogenic trapping is required for the analysis of thermally labile compounds but is more effected by coextration of water vapor than most sorbent traps. The purge gas is led through an efficient low temperature (ca. -15°C) condenser where most of the water is frozen out. The purge gas continues through a heated compartment into a cryogenic trap consisting of a length of fused-silica capillary tube cooled by liquid nitrogen. The capillary trap may be empty, packed, or coated with stationary phase depending on the analytes to be trapped. A high purge flow is achieved without disturbing the column flow by a splitter in the column oven, where the purge flow is vented through a solenoid valve. The solenoid valve is closed at the completion of sampling for the introduction of the trapped volatile organic compounds by thermal desorption into the separation column (section 3.7.2).

Water management is a more significant problem for dynamic headspace than static headspace sampling methods. Static headspace methods use a relatively small headspace gas volume while dynamic headspace requires trapping of a larger water saturated purge volume. Water removal techniques have included permeation and condensation devices (e.g. Figure 3.14). During typical purge-and-trap sampling conditions 10-80 mg of water may be coextracted. Diffusion of water through a permeable membrane (e.g. Nafion) provides an efficient water removal method but is accompanied by the partial or complete loss of light polar and oxygenated compounds (e.g. alcohols, ketones, aldehydes) [142,165,170]. As interest grew in the analysis of

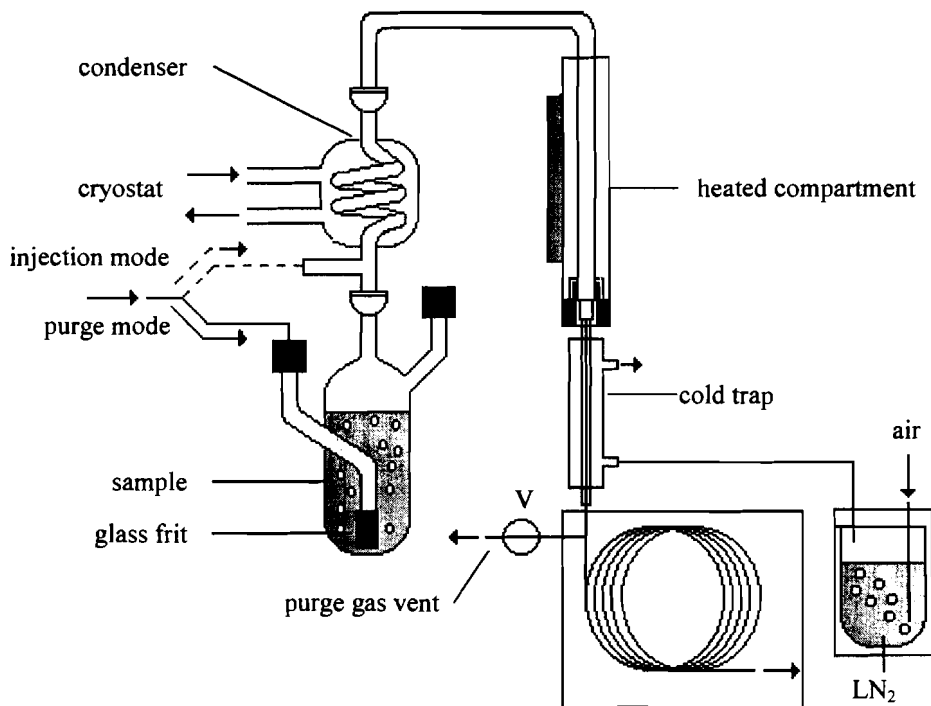


Figure 3.14. Schematic diagram of a purge-and-trap apparatus for gas extraction and cryogenic trapping of volatile organic compounds from water. (From ref. [142]; ©Elsevier).

these compounds, permeation dryers became less popular, leaving condensation as the primary method of water removal. Thermoelectric as well as gas and liquid cryostats are used for cooling the condensation zone, which need not be chilled to temperatures below -25°C . Condensation can lower water levels to as little as 0.063 mg/min without trapping the volatile compounds typically analyzed by purge-and-trap. Water removal during static headspace analysis, if required at all, is usually achieved by using a short trap containing a support coated hygroscopic salt (e.g. LiCl) that can be reactivated by thermal cycling [171].

3.7.4 Pyrolyzers

Pyrolysis is a technique that uses heat to transform macromolecules into a series of lower molecular weight volatile products characteristic of the original sample. The initiation reactions involved in the pyrolysis of most organic materials are the result of free radical reactions initiated by bond breaking or by the unimolecular elimination of simple molecules such as water or carbon dioxide [172-175]. The products of pyrolysis are identified by gas chromatography, often in combination with mass spectrometry,

when more information than a comparative fingerprint (pyrogram) of the sample is required. The samples most commonly analyzed by pyrolysis gas chromatography tend to be complex, involatile, highly polar, high molecular weight and unsuitable for analysis using conventional gas chromatographic techniques. Intractable samples, such as rubbers, textiles, paints, bio-, geo- and synthetic polymers, plant materials, coal, sediments and bacteria are natural choices for this technique [172,176-180]. However, the application of pyrolysis gas chromatography is not limited to such samples alone; small molecules, such as drugs in biological fluids and solvents and monomers entrained in polymers can also be determined by pyrolysis techniques.

Two main approaches have emerged as the preferred means to induce thermal decomposition of the sample by delivering heat at a constant temperature (continuous mode), or by applying a well-defined heating pulse (pulse mode) to the sample. Final temperatures between 500-800°C are employed in most analytical work. Laser disintegration techniques are more complex and expensive [181,182]. They are usually reserved for special applications. The PTV injector (section 3.5.4) combined with a cryogenic trap is a suitable device for the multi-step, thermal desorption-pyrolysis characterization of geochemical and petroleum samples by gas chromatography [71,183]. Powdered rock samples (< 2 g) are housed in the injection liner and subjected to step-programmed isothermal treatment at 200, 400 and 600°C.

Continuous mode pyrolyzers include a wide range of resistively heated tube furnaces that can be operated on- or off-line; the latter with solvent or cryogenic trapping to collect the pyrolysis products for subsequent separation by gas chromatography [172,177]. The sample is usually introduced into the furnace by a special syringe with a needle within a needle design (the inner needle has a groove into which the sample is placed) or by a boat, dropped or pushed, into the heated zone. On-line continuous pyrolyzers are mounted directly onto the gas chromatographic inlet with the carrier gas routed through the microfurnace. A thermocouple is used to monitor the tube wall temperature ($\pm 1^\circ\text{C}$). The principal disadvantage of continuous mode pyrolysis is that heat transfer to the sample is relatively slow and the wall temperature of the microfurnace is usually much higher than the temperature of the sample. The pyrolysis products, therefore, migrate from the sample to hotter regions of the pyrolyzer where additional reactions may be initiated to produce secondary products. This process is different from that occurring in pulse-mode pyrolyzers and may result in either the formation of different products or the formation of the same products, but with a different distribution. Microfurnaces have a relatively large dead volume and are usually operated at high flow rates (e.g. 100 ml/min) in combination with a split injector. The high gas flow rate also helps to minimize the formation of secondary products by reaction at the hot column wall.

Pulse-mode pyrolyzers include resistively heated electrical filaments or ribbons and radio frequency induction-heated wires [172,177-180,184]. The filament or ribbon-type pyrolyzers are simple to construct and typically consist of an inert wire or ribbon of high electrical resistance (e.g. Pt or Pt-Rh alloy) connected to a high-current power supply. The filament is housed in a heated low dead volume chamber swept by the

carrier gas and connected directly to the gas chromatographic inlet. The temperature of the filament is monitored by changes in its resistance or by a photodiode to provide accurate control of the heating rate (up to 30,000°C/s) and the final set point temperature and time. The filament pyrolyzer can also be continuously or stepwise temperature programmed, to enhance the diagnostic information about the sample. The filament is shaped for convenience of sampling and may be a flat strip, grooved strip, wire, or coil. In the case of the coil, a small quartz tube containing the sample is inserted into the coil and subsequently heated by it. Filament or ribbon-type pyrolyzers are difficult to automate since each sample must be delivered to the same filament. In a Curie-point pyrolyzer, an oscillating current is induced in the pyrolysis wire or foil by means of a high-frequency coil. The eddy currents induced in the wire conductor cause its temperature to rise rapidly until a specific temperature is reached, its Curie point, at which the ferromagnetic materials become paramagnetic, and the absorption of energy ceases. The temperature then stabilizes at the Curie-point temperature. This technique provides a highly reproducible pyrolysis temperature, since the Curie-point temperature of a ferromagnetic material depends only on the composition of the alloy. By selecting wires of different composition temperatures in the range 300 to 1000°C can be obtained. The pyrolysis chamber is swept by the carrier gas and independently heated to prevent condensation of pyrolysis products within the chamber. The pyrolyzer is usually connected directly, or via a switching valve, to the gas chromatograph. The pyrolyzer must be designed to allow easy insertion of the ferromagnetic wire or foil into the pyrolysis chamber. Because the sample holder is inductively heated automated operation is facilitated [185,186].

Since in the pulse-mode pyrolyzers the sample and heat source is in intimate contact, heat transfer is rapid and temperature gradients within the sample should be absent. The primary pyrolysis products are quenched as they expand rapidly into the cooler regions of the pyrolyzer, diminishing the possibility of secondary product formation. Heating rates are rapid (milliseconds), reproducible, and controllable by adjusting the filament-heating current or alloy composition and radio frequency energy of the Curie-point pyrolyzer. The reproducibility of individual pyrograms depends on the sample size and homogeneity, the temperature profile of the pyrolyzer, absence of catalytic transformation on metal surfaces and the maintenance of uniform conditions for transferring the pyrolysis products to the column [178,187,188]. For reproducible pyrolysis the sample should be applied as a thin, uniform film from solution to provide a final sample size of 5 to 100 µg of organic material after solvent evaporation. For those samples where no suitable solvent exists other approaches are required. Finely ground powders can be applied as a suspension and dried. Easily melted samples can be applied by dipping and other solid samples by encapsulation in a pyrofoil or by wrapping the wire around the sample. Differences in the temperature profile, rise time and equilibrium temperature can be a major cause of poor reproducibility between different types of pyrolyzers and between different laboratories using the same type of pyrolyzer. Curie-point pyrolyzers may suffer from small temperature variations due to differences in alloy composition of the wire conductor, sample loading and positioning of the conductor

within the induction coil, and fluctuations in the radio frequency energy. Because of the large dead volume of the pyrolyzer and the finite time needed for complete pyrolysis, the direct coupling of the pyrolyzer to open tubular column gas chromatography raises some additional problems [188-190]. A split-type interface allows a high flow rate of 20-100 ml/min of gas through the pyrolyzer to rapidly sweep the pyrolysis products to the gas chromatograph with only a small portion (1-5%) of this flow entering the column. Since the sample size pyrolyzed is usually larger than the column capacity, and splitting of the hot vapors is expected to occur without discrimination, this is rarely a problem. When splitting is not appropriate, or column cold trapping fails to provide adequate band focusing, cryogenic trapping followed by rapid thermal desorption is used. The advent of electronic pressure control allows pressure pulse techniques to be used to rapidly transport the pyrolysis products to the column followed by a lower flow rate to optimize the column separation [190].

3.8 COUPLED-COLUMN GAS CHROMATOGRAPHY

Coupled-column separations employ several (usually two) columns of different capacity or selectivity connected in series via a suitable interface [191-194]. Using the interface to adjust the relative residence time of the sample components in two columns of different selectivity provides a powerful approach for method development known as selectivity tuning (section 2.4.2). Using a modulator as an interface fractions from the first column can be continuously transferred and separated on the second column as a series of secondary chromatograms that can be assimilated into a two-dimensional separation. This technique is known as comprehensive two-dimensional gas chromatography (section 3.8.2) and is capable of generating the very high peak capacities needed for separating complex mixtures [193]. Multidimensional gas chromatography is the term used to describe separations based on the transfer of just part of the separation from the first column (heartcut) to the second column, possibly in combination with cryogenic trapping.

3.8.1 Multidimensional Gas Chromatography

Multidimensional gas chromatography employs a series of procedures that provide improved separations for sections of a one-dimensional chromatogram by their transfer and independent separation on a second column. Instrumentation for multidimensional gas chromatography is somewhat complex, and if multiple fractions from the first column are to be transferred and separated on the second column, the process can be time consuming. The early interest in multidimensional gas chromatography has not translated into general acceptance in routine analytical laboratories. Current use is restricted to a small number of research centers. Principal applications include the detailed fingerprinting of petroleum products, the separation of polychlorinated biphenyl congeners and the separation of enantiomers in flavor and food technology

[194,195]. One-dimensional column separations, even with mass selective detectors, fail to provide adequate separations in these cases justifying the additional effort to develop multidimensional separations.

Heartcutting is the most common procedure in multidimensional gas chromatography. It is used to isolate and transfer selected sections of the first column chromatogram to the second column. Backflushing is often used in conjunction with heartcutting to reduce the separation time for the total sample. Sample components with large retention factors on the first column can be eluted in a reasonable time by reversing the direction of the carrier gas flow through the column. The backflushed components can be eluted through a detector for analysis or simply vented to waste. Foreflushing is used to remove solvent or major components from the sample where these would have a deleterious effect on the operation of the second column or the detector used to record the separation. For example, large volume injection for trace analysis or the detection of derivatized analytes in the presence of excess reagents.

The interface in multidimensional gas chromatography must provide for the quantitative transfer of the effluent from one column to the next without altering the composition of the transferred sample or degrading the resolving power of the second column [34,191-196]. Interfaces employing switching valves or pneumatic switches are commonly used for this purpose. Pneumatic switches, also commonly referred to as Deans' switches, are based on the balance of flow at different positions in the chromatographic system. The flow direction is adjusted by pressure regulation using valves and associated components located outside the column oven. Pneumatic switches can easily handle the widely varying flows that exist in interfacing columns of different types (e.g. packed and open tubular columns) [191,195,197]. They are more difficult to optimize than valve interfaces but are free of the problems associated with the use of valves. Electronic pressure control provides improved operation and keyboard control of all operating parameters when using pneumatic switches [198].

Switching valves (section 3.4.2) represent the simplest approach to control the flow of carrier gas between columns [34,199-201]. Since the heated valve forms part of the sample passageway, the valve must be chemically inert, gas tight at all temperatures, have small internal dimensions, and operate without lubricants. During passage through the valve the sample comes into contact with the metal valve body (e.g. stainless steel) and elastomer material (e.g. fluorocarbon-filled crosslinked polyimide) used to machine the rotor. Sample residence times are typically very short (few μ s). Separate thermostating of the valve becomes necessary using a dual oven configuration in which the valve is located between the two ovens and when rapid temperature program operation is used with a single oven configuration. Switching valves are easily automated using pneumatic or electric actuators and are useful for coupling columns operated at similar flow rates for separations obtained at moderate temperatures. Conflicting reports of the decomposition or tailing of polar and labile compounds on hot valve surfaces requires a cautionary approach [194].

An intermediate cryogenic trap is essential whenever a band focusing mechanism is required as part of the transfer process [194-197,202-204]. Examples include sample

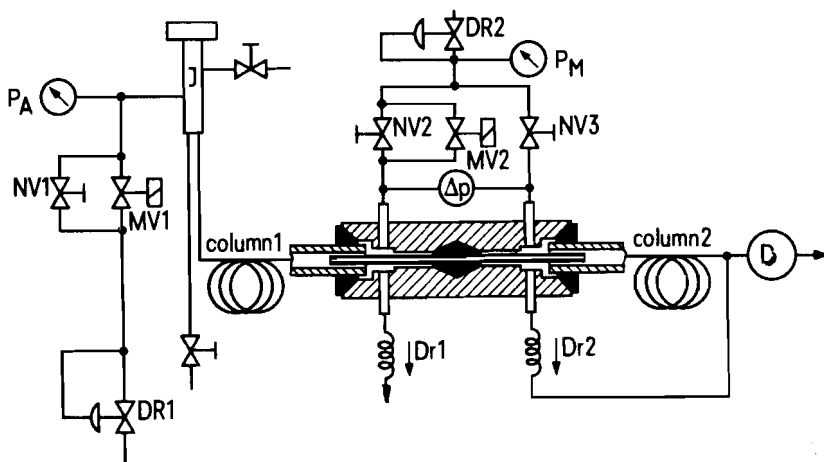


Figure 3.15. Schematic diagram of a dual oven two-dimensional gas chromatograph with live switching between two capillary columns. DR = precision pressure regulators; NV = needle valves; P = pressure gauges; Dr = flow restrictors; and D = detectors.

transfer from packed to open tubular columns, accumulation of fractions from the same or sequential separations, and accurate determinations of retention index values on the second column. The design and operation of cryogenic traps are described elsewhere (section 3.7.2). Multiple cryogenic traps connected in parallel provide increased productivity by facilitating the accumulation and storage of several fractions from the first column for sequential separation on the second column [205,206].

A number of kits are available for the conversion of any gas chromatograph to a multidimensional gas chromatograph [194,198]. A two-oven configuration allows greater operational flexibility since the temperature regime for the two columns can be optimized independently. To simplify installation all the pneumatic and time sequencing components are usually located outside the column oven. Inside the oven is positioned the cold trap and either a valve or connecting T-piece for pneumatic switching. Diversion of a small flow of carrier gas from the first column to a detector allows accurate locations for the heartcuts to be made.

A schematic diagram of the switching components for a purpose-built, two-oven multidimensional gas chromatograph employing live switching for sample transfer is shown in Figure 3.15 [34,194,195]. The critical component of the system is the double T-piece with tube connections for adjusting pressures and a platinum/iridium or fused silica capillary for the connection of the two capillary columns. The direction of flow through the double T-piece is controlled by slight pressure differences applied between its ends. The T-piece is constructed in such a way that any gas flowing from one end to the other must pass through the connecting capillary, which is inserted loosely into both columns. Gas flow from the first column can be directed to a detector via a restrictor, or through the second column and then to a detector. Moreover, the first column can

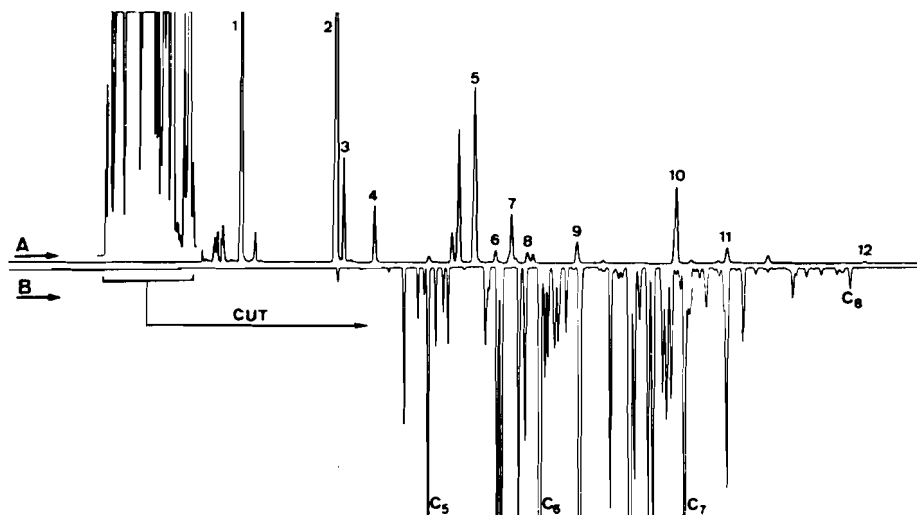


Figure 3.16. Two-dimensional separation of the components of a coal derived gasoline fraction using live switching. Column A was 121 m open tubular column coated with poly(ethylene glycol) and column B a 64 m poly(dimethylsiloxane) thick film column. Both columns were temperature programmed independently taking advantage of the two-oven configuration. Peak identification: 1 = acetone, 2 = 2-butanone, 3 = benzene, 4 = isopropylmethylketone, 5 = isopropanol, 6 = ethanol, 7 = toluene, 8 = propionitrile, 9 = acetonitrile, 10 = isobutanol, 11 = 1-propanol, and 12 = 1-butanol. (From ref. [195]; ©Elsevier).

be backflushed by activating solenoid valves in the carrier gas and T-piece flow lines. An example of the live switching technique for the separation of the components of a coal derived gasoline fraction is shown in Figure 3.16. Using a polar column as the first column circumvents the overlapping of components of different polarity but similar boiling point. The selectivity of this column for the resolution of the hydrocarbon isomers is inadequate, however, and therefore, the hydrocarbon fraction was heartcut to a non-polar column for separation. The total separation time remains acceptable since both separations are performed simultaneously.

3.8.2 Comprehensive Two-Dimensional Gas Chromatography

When only a few compounds are of interest in a complex sample, multidimensional gas chromatography has the advantage that only a small number of selected fractions have to be transferred to the second column. The number of heartcuts that can be transferred before peaks from one fraction interfere with those of another limits multidimensional gas chromatography when more compounds are of interest. Although sequential injections and heartcuts, or several parallel traps to store fractions prior to sequential separation on the second column can be used, these techniques are likely to be time consuming and comprehensive two-dimensional gas chromatography is likely to provide better results in less time [141,192,207-210]. On the other hand,

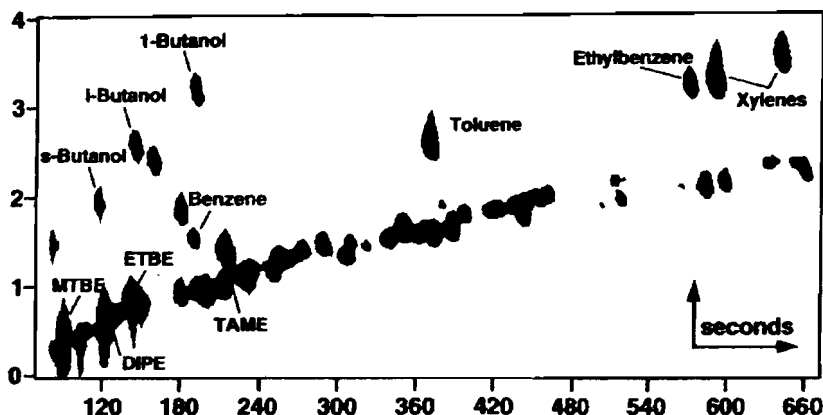


Figure 3.17. Contour plot of a separation of ethers, alcohols, alkylaromatics and saturates by comprehensive two-dimensional gas chromatography. (From ref. [168]; ©Elsevier).

multidimensional gas chromatography is a mature technique while comprehensive two-dimensional gas chromatography is at an early stage in its evolution.

Comprehensive two-dimensional gas chromatography employs two columns connected in series with total transfer of all sample components from the first column to the second column as a series of pulses. Each pulse is separated sequentially and individually on the second column resulting in a large number of secondary chromatograms. A modulator, situated close to the junction of the two columns, is required to create the sharp equidistant pulses. The dimensions of the second column are selected to allow very fast separations such that enough secondary chromatograms are produced during the elution of each peak from the first column that the first column chromatogram can be reconstructed with fidelity. The secondary chromatograms are usually stacked side by side to form a two-dimensional plot with the first dimension representing the retention time on the first column and the other, the retention time on the second column. The most convenient way to visualize these chromatograms is as contour plots, where peaks are displayed as spots on a plane using colors and/or shading to indicate the signal intensity, Figure 3.17 [207,211-214]. Each peak must be integrated over both dimensions to give a peak volume, which is directly proportional to the quantity of substance forming the peak [215]. This involves determining the beginning and ending points of the peak on both columns and then, following baseline correction, summing the values of the data points within the selected space. Multivariate techniques, like the generalized rank annihilation method (GRAM), can use the inherent structure in two-dimensional data to mathematically separate and quantify resolved and incompletely resolved signals [215]. GRAM compares the data of a sample and calibration standard and extracts the individual signals of the chemical components from that of interference and noise. In this method, the GRAM extracted signals are used to determine the relative amount of each analyte of interest in the sample. Run-to-run variation of retention times in both di-

mensions, a frequent problem in data analysis of comprehensive two-dimensional chromatograms, can be corrected through software alignment [216]. ANOVA-based feature selection followed by principal component analysis for pattern recognition classification has been applied successfully to data obtained from two-dimensional comprehensive separations [217]. In terms of automated data analysis of two-dimensional comprehensive separations considerable challenges remain. The large amount of data generated makes this an important issue for the wider use of these methods.

By arranging for a low correlation of the separation mechanism on the two columns a large peak capacity (theoretically equal to the arithmetic product of the two one-dimension separations) is obtained using comprehensive two-dimensional gas chromatography. It is this large peak capacity that accounts for the greater separation potential of comprehensive two-dimensional gas chromatography compared with single column separations. An additional benefit of modulating the separation is a several-fold improved signal-to-noise ratio compared with a normal separation [213,218,219]. Component identification should be more reliable in comprehensive two-dimensional gas chromatography because each substance has two identifying retention values rather than one. Two-dimensional separations are also more structured leading to recognizable patterns characteristic of the sample type. Broad classes of compounds, as indicated in Figure 3.17, appear in horizontal bands in a comprehensive two-dimensional separation (sometimes called a roof tile effect) providing a useful interpretation aid.

The key instrumental component for generating a comprehensive two-dimensional separation is the modulator. The modulator must be able to accumulate material eluted from the first column during the time that the separation on the second column occurs and then transfer the trapped material as a narrow band to the second column. The processes of analyte accumulation (focusing) and transfer to the second column (injection) must be reproducible, repeatable and non-discriminating with respect to sample properties. Early success was achieved using two-stage thermal modulators prepared by coating a portion of a capillary column with an electrically conductive paint or wrapping the column with a copper wire coil [220]. The modulation capillary was installed between the two separation columns in a cold zone outside the column oven. Compounds were accumulated by cold trapping in the coated modulator column and injected into the second column by thermal desorption achieved by applying a current pulse through the conductive paint layer or wire coil. Poor durability of these modulators resulted in their replacement by thermal modulators using a moving slotted heater, longitudinally modulated cryogenic trap or cryo-jets. Differential flow modulation provides an alternative approach to thermal modulation and is simpler to implement.

The moving slotted heater interface, Figure 3.18, mechanically sweeps a slotted heater over a short modulator capillary connected at either end to the separation columns [207,213,221-223]. The modulator capillary is coated with a thick film of stationary phase terminated in an uncoated zone from which the trapped components are rapidly desorbed when the slotted heater passes over them. The heater is paused for about 0.5 s at the end of each revolution to facilitate sample desorption and transfer.

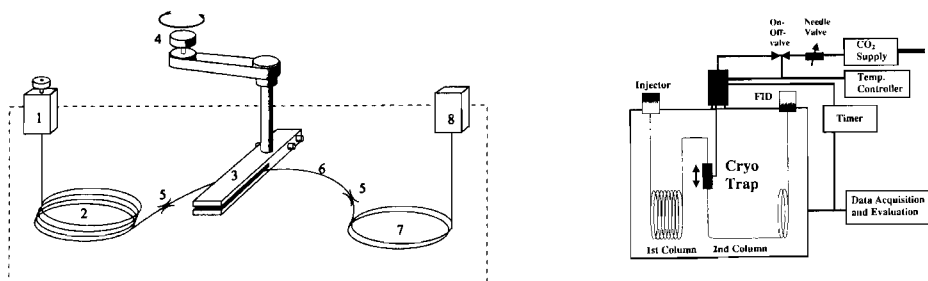


Figure 3.18. Thermal modulators for comprehensive two-dimensional gas chromatography. The slotted moving heater (left) and longitudinally modulated cryogenic trap (right). Components: 1 = injector; 2 = first dimension column; 3 = slotted heater; 4 = stepper motor; 5 = press fit connector; 6 = thick film modulator capillary; 7 = second dimension column; 8 = detector. (Adapted from ref. [168] and [225]; ©Elsevier).

Sample components are accumulated by cold trapping at the column oven temperature. Rapid thermal desorption requires a temperature difference of about 100°C between the maximum column oven temperature and the temperature of the slotted heater. This requirement effectively establishes an upper temperature limit for the column oven and therefore the volatility range of the compounds that can be separated.

The longitudinally modulated cryogenic trap interface consists of a small cryogenic trap moved with an oscillating longitudinal motion over a section of the second capillary column, Figure 3.18 [141,210,223-225]. The small movements of the trap are controlled by a pneumatic or electrical drive with a maximum oscillation frequency of 5 Hz. The migration of solutes entering the cryogenic trap are quickly arrested by cold trapping in the stationary phase, effectively focusing the accumulating material into a narrow band. Displacement of the trap from the accumulation zone exposes the focused band to the column oven temperature releasing the material as a narrow pulse. A short pause of about 0.5 s is programmed at the end of each cycle to facilitate remobilization of the sample. The cryogenic trap is typically maintained at a constant temperature. Recent studies suggest, however, that operation with a constant flow of coolant relieves occasional problems associated with poor peak shapes and incomplete remobilization of analytes of low vapor pressure and the modulation of column bleed (and other system impurities) [225]. Modulated column bleed can appear as interfering peaks in the second dimension chromatogram. The longitudinally modulated cryogenic trap has a wider temperature operating range than the moving slotted heater since thermal desorption occurs at the column oven temperature without additional heating. Both modulators are capable of high frequency operation and produce sample pulses with initial bandwidths less than 100 milliseconds and typically 20-50 milliseconds.

The current practice of comprehensive two-dimensional gas chromatography is dominated by systems using thermal modulators based on the slotted heater or longitudinally modulated cryogenic trap. The cryo-jet modulator is an attempt to design a stationary-thermal modulator [226]. The cryo-jet modulator consists of two fixed-position nozzles positioned close to each other. These nozzles spray alternating pulses

of carbon dioxide coolant onto a section of the second dimension capillary column. The cryo-jets trap and focus fractions from the first dimension separation, which are subsequently remobilized by heat from the surrounding oven air. Modulation times as short as 0.1 s are possible using this promising approach.

Differential flow modulation employs a multiport diaphragm valve with sample loop located in the chromatographic oven, or separate thermostatted oven box, to couple the two separation columns [227-229]. The effluent from the first column is collected in the sample loop and then transferred to the second column. During the transfer process, the effluent from the first column is vented to waste. Thus, sample transfer from the first to the second column is periodic, but not quantitative, and is described by a duty cycle. Typically, the valve is in the collect position for about 80-90% of the time and the switching frequency is about 1 Hz. These conditions allow, for example, filling of the sample loop for 0.9 s with a 0.1 s flush of the loop onto the second column.

Method development requires a different and more complex approach than the optimization of conventional one-dimensional gas chromatographic separations [210,230-232]. The overall separation is influenced by the column properties (length, diameter, phase ratio, stationary phase type), the carrier gas velocity, temperature regime for both columns and the modulation frequency. Several of these parameters cannot be changed independently. The crucial practical problem is to obtain optimum matching between the modulator frequency and the speed of the second column separation. Maximum separation performance requires that each peak from the first dimension is modulated into at least three to four fractions and that separations on the second column are about 50 times faster than on the first column. If the second column separation is too slow relative to the first column then resolution in the first dimension is sacrificed. If the second column separation is too fast relative to the first column, the total separation time is increased and poor resolution is possible on the second column. In general practice typical second column separations are of 3-5 s duration requiring 20-30 s peak widths at base for the first column. This requirement is met by slowing down the first column separation by using thick film columns and operating conditions that result in greater peak broadening than customary for conventional separations. Although a longer second dimension column will generally lead to a better separation, wrap-around may cause otherwise separated components to co-elute in the two-dimensional chromatogram. Wrap-around is observed when the second dimension retention time for an analyte exceeds the modulation period. Since data are transformed into matrix format based on the modulation cycle, the analyte peak will be located at a false position in the two-dimensional chromatogram, possibly in a region already occupied by other sample components.

Although optimization was achieved by largely empirical means, a number of impressive separations of complex mixtures (petroleum products [211,214,233], essential oils [234], polychlorinated biphenyls [235], low-volatility chlorinated compounds [236] and fatty acid esters [212]) have been published. In addition, the possibility of interfacing two-dimensional comprehensive gas chromatography with time-of-flight mass spectrometry has been demonstrated [212,237]. This later combination provides a very powerful tool for target compound analysis by combining retention information on two

columns with structural information from mass spectrometry, as well as providing for conventional structural elucidation of unknown compounds.

3.9 COLUMN CONNECTORS AND FLOW SPLITTERS

Methods for connecting open tubular columns of the same or different diameter are important for several common chromatographic procedures, such as connecting retention gaps to separation columns, connecting early solvent vapor exits to precolumns, fabrication of column flow splitters and Deans' switches, and for constructing series-coupled gas chromatographic systems [40]. The most common methods of making leak-tight, low-dead volume connections for open tubular columns are press-fit connectors [238], butt connectors [239], heat shrinkable PTFE joints [240,241], and various procedures using glues and guiding tubes [40]. Press-fit connectors are the most popular and easy to use. Press-fit connectors are prepared using short glass tubes with a slight restriction at their center. The fused-silica capillary tube is pressed into the tapered seat of the connection tube with the thin polyimide capillary outer coating forming a seal with the inside wall of the connection tube. After heating the connection to over 200°C a permanent seal is formed. Press-fit connectors can be reused after vaporizing the polyimide seal by heating in a furnace to 550°C [242]. Press-fit Y-connectors are commonly used as flow splitters and for the construction of Deans' switches and multidimensional gas chromatography systems [243,244].

Low-mass butt connectors are available from many vendors and achieve a stable joint using standard ferrules and screw-thread fittings. They are straightforward to use but problems may arise from distorted peaks, ghost peaks, and memory effects associated with interactions with polymeric or carbon ferrules; decomposition or alterations caused by interactions on hot metal surfaces; and from the lack of a simple visible test to check for misalignment or breakage of the capillary tubes. Ferrule materials are typically made of polyimide, polyimide and graphite composites or graphite and have largely replaced other polymers. There is no perfect ferrule material. Polyimide ferrules are hard and require significant force to form a seal. Graphite is soft and forms finger tight seals but is mechanically weak and sometimes releases particles that lodge in various places in the chromatographic system affecting performance. Polyimide and graphite composites provide intermediate flow, adhesion and stability properties. All possess good temperature stability.

Heat shrinkable PTFE tubing is one of the oldest methods of connecting fused silica or glass open tubular columns. Connections are easy to make by simply aligning the two capillary tubes inside a 10-15 mm length of shrinkable PTFE tube and warming the shrink tube with a small burner until it collapses over the capillary tubes. A more stable connection is prepared by heat shrinking a second PTFE tube of larger internal diameter over the first or by sliding the connection into a glass sleeve. The problems with PTFE connections include poor mechanical stability above about 250°C and permeability to sample components and atmospheric oxygen above about 200°C [40,240]. Large

solvent injections may cause tailing of the solvent front into the chromatogram due to the sorption and slow release of the solvent by the PTFE polymer.

Flow splitters are used to split injections between parallel columns or to monitor separations with parallel detectors to enhance the information content of the chromatogram, or to generate substance-characteristic detector response ratios, which aid compound identification. Ideally, a flow splitter should provide a fixed split ratio that is independent of flow rate, temperature and sample volatility. It should also minimize band broadening, be chemically inert, and for convenience, provide some mechanism for adjusting the split ratio over a reasonable range (e.g. 1:1 to 1:100) [244-246]. For packed columns, T-splitters with fixed or valve-adjustable split ratios are generally used [247,248]. Dead volume effects are not usually a problem at packed column flow rates, although changes in the split ratio with temperature and the constancy of the split ratio with samples of different volatility may be. At the low flow rates used with capillary columns, dead volume effects can become significant unless the total volume of the splitter is reduced to the absolute minimum and all passageways are completely swept by carrier and/or makeup gas. Flow splitters for open tubular columns have been fabricated from glass-lined stainless steel, platinum/iridium, and glass capillary tubing [244-246,249-251]. These may be in the form of simple T- or Y-splitters for dual detector operation or more complex manifolds for multiple detection and column switching. Most laboratory made flow splitters are fabricated from glass because it is easy to work with and relatively inert.

3.10 DETECTORS

Numerous methods have been described for the on-line detection of organic vapors in the carrier gas flowing from the column in gas chromatography [1-4,7,252-254]. Those detectors that have reached commercial maturity and are widely used form the backbone of this section. The important spectroscopic detectors (e.g. mass spectrometry, infrared, nuclear magnetic resonance, etc.) are covered in chapter 9. Other detectors such as radiochemical, olfactometry and electroantennography will be mentioned only briefly. They are generally confined to specialized laboratories with specific application needs. Radiochemical detectors are used to study the distribution and fate of volatile organic compounds in complex systems using radiolabelled (e.g. ^3H , ^{14}C , etc.) compounds [255,256]. Compounds in the column effluent are oxidized or reduced over a metal catalyst in a high-temperature (e.g. 700°C), flow-through microfurnace. The products formed (e.g. water, methane, carbon dioxide, etc.) flow through a gas proportional counter where those products resulting from the decomposition of radiolabelled compounds are registered. Olfactometry is used for the detection of odor-active chemicals with a purpose designed "sniffer" [257,258]. Odor evaluation requires a trained individual and a well-ventilated environment. The hot carrier gas is usually diluted with humidified air and transferred to a port or mask for assessment. Splitting the column flow between a standard detector and the "sniffer"

provides a correlation between chromatographic peaks and odor producing compounds. Electroantennography is a neurophysiological technique that allows an insect's reaction to chemical stimuli to be monitored [24]. A pair of electrodes is connected to the insect's antenna and the diluted carrier gas passed over the insect. A response, measured as a depolarization across the antenna, generates a voltage in the electrodes that is amplified and continuously recorded. Olfactometry and electroantennography provide powerful examples of the sensitivity of biological systems to low concentrations of chemicals that may go undetected by conventional gas chromatographic detectors.

The principal methods of detection in gas chromatography can be grouped under four headings based on the physical principle of the detection mechanism: ionization, bulk physical property, optical and electrochemical detectors. Further division into universal, element-selective and structure-selective detectors are possible based on the characteristics of the detector response. The flame ionization and thermal conductivity detectors respond to the presence of nearly all organic compounds in the column effluent and are considered to be general or (near) universal detectors. Other detectors respond only to the presence of a particular heteroatom (e.g. flame photometric, thermionic ionization, or atomic emission detectors) and are element-selective. Or they respond only to a structural feature related to the bond energy of two or several atoms in a molecule (e.g. electron capture and photoionization detectors) and are structure-selective. Useful selective detectors are characterized by a wide response range, covering several orders of magnitude, providing for a measure of discrimination between compounds possessing the feature to which the detector responds and other compounds lacking that property. The selectivity of a detector can be described according to circumstance by the ratio of the detector response to two different compounds, compound classes, heteroatoms, etc. General performance characteristics and figures of merit for chromatographic detectors were discussed elsewhere (section 1.8.1).

3.10.1 Ionization Detectors

At the temperatures and pressures generally used in gas chromatography the common carrier gases employed behave as perfect insulators. In the absence of conduction by the gas molecules themselves, the increased conductivity due to the presence of very few charged species is easily measured, providing the low sample detection limits characteristic of ionization based detectors [259]. Examples of ionization detectors in current use include the flame ionization detector (FID), thermionic ionization detector (TID), photoionization detector (PID), the electron-capture detector (ECD), and the helium ionization detector (HID). Each detector employs a different method of ion production, but in all cases the quantitative basis of detector operation corresponds to the fluctuations of an ion current in the presence of organic vapors.

3.10.1.1 Flame Ionization Detector

A nearly universal response to organic compounds, low detection limits, long term stability, simplicity of operation and construction, low dead volume, fast signal

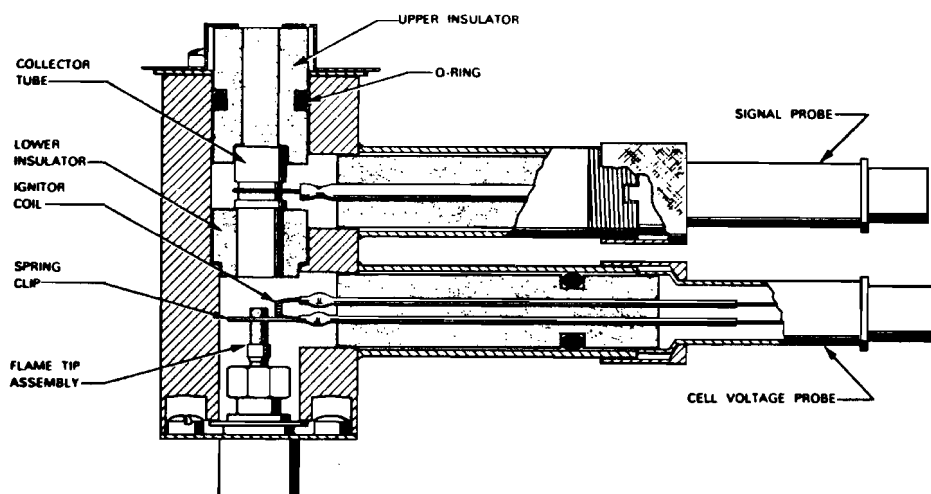


Figure 3.19. Cross-sectional view of a flame ionization detector. (©Varian Associates).

response, and exceptional linear response range have contributed to the flame ionization detector (FID) being the most popular detector in current use. Only the fixed gases (e.g. He, Xe, H_2 , N_2), certain nitrogen oxides (e.g. N_2O , NO, etc.), compounds containing a single carbon atom bonded to oxygen or sulfur (e.g. CO_2 , CS_2 , COS, etc.), inorganic gases (e.g. NH_3 , SO_2 , etc.), water and formic acid do not provide a significant detector response. Minimum sample detection limits corresponds to about 10^{-13} g carbon/s with a linear response range of 10^6 to 10^7 .

The FID response results from the combustion of organic compounds in a small hydrogen-air diffusion flame, Figure 3.19. The carrier gas from the column is mixed with hydrogen and burned at a narrow orifice in a chamber through which excess air is flowing. A cylindrical collector electrode is located a few millimeters above the flame and the ion current is measured by a small voltage established between the jet tip and the collector electrode. Under typical operating conditions background currents of 10^{-14} to 10^{-13} Amperes, increasing to 10^{-12} to 10^{-5} Amperes in the presence of an organic vapor, are common. The small ion currents are amplified by a precision electrometer. Detector performance is influenced primarily by the ratio of air-to-hydrogen-to-carrier gas flow rates, the type (thermal conductance) of the carrier gas and detector geometry [254,260]. The optimum response plateau is usually fairly broad, permitting operation over a rather wide range of gas flow rates without incurring a large penalty in diminished response. The upper end of the linear response range is influenced primarily by the flame size, the bias voltage at the collector electrode and detector geometry.

The mechanism of ion production in flames is complex and only partially understood [261]. The temperature and chemical composition of the flame is not uniform. In the hottest part of the flame a temperature of about 2000°C is reached. The flame also contains a large concentration of radicals (H^\bullet , O^\bullet , and OH^\bullet , etc). The ionization

efficiency of the flame is not particularly high, perhaps a few ions per million molecules are formed. This is consistent with the fact that the thermal energy of the flame is too low to explain the production of ions. It is generally believed that ion formation results from chemical ionization of CHO^* produced by reaction of O^\bullet and CH^\bullet



The ionization process is expected to be a first order reaction, explaining the linear response of the FID. The ionization mechanism would be placed in a low probability reaction pathway, explaining the low ion yield. Two steps are thought to be important in the above process: radical formation, requiring the absence of oxygen, and chemical ionization of radicals formed by atomic or molecular oxygen excited states. The degradation of organic compounds in the FID flame most likely results from attack by hydrogen atoms causing fission of carbon-carbon bonds. Fission of unsaturated bonds proceeds after hydrogenation. The hydrocarbon radicals produced by fission are unstable and undergo a cascade of fast fractionation and hydrogenation reactions so that all carbon atoms are converted to methane. Methane has the strongest carbon-hydrogen bonds of all hydrocarbons and is the logical end point for the reaction of organic compounds with excess hydrogen atoms. Consequently, each carbon atom capable of hydrogenation yields the same signal, and the overall FID response to each substance is proportional to the sum of these “effective” carbon atoms. The FID response is highest for hydrocarbons, being proportional to the number of carbon atoms, while substances containing oxygen, nitrogen, sulfur, or halogens yield lower responses, depending on the characteristics of the carbon-heteroatom bond and the electron affinity of the combustion products. The lower response is due to competition between hydrogenation of the carbon-heteroatom bond and hydrogen abstraction with formation of neutral species (e.g. CO, HCN) which are poorly ionized in the flame. The effective carbon number for a particular compound can be estimated by summation of the various carbon and heteroatom contributions, Table 3.3, which in turn can be used to predict relative response factors with reasonable accuracy [262-264]. Response factors can also be predicted from quantum chemical descriptors using quantitative structure-property relationship models [264,265]. This simplifies the quantification of complex mixtures where structures from mass spectrometry may be available but not standards

With relatively minor modifications the FID can be operated as an element-selective detector. The thermionic ionization detector (section 3.10.1.2) is the most important of these. The hydrogen atmosphere flame ionization detector (HAFID) can be made selective to organometallic compounds containing, for example, Al, Fe, Sn, Cr, and Pb [254,266]. The detector employs a hydrogen-oxygen flame burning in a hydrogen atmosphere doped with a reagent such as silane to improve its response consistency. Conversely doping with an organometallic compound enables the HAFID to function as a silicon-selective detector. The remote flame ionization detector (RFID) is able to detect long-lived negative ions produced from the combustion of Pb, Sn, P or Si compounds in an organic fueled (methane) flame [267]. The RFID differs from

Table 3.3
Contributions of structure to the response of the flame ionization detector

Atom	Type	Effective carbon number
C	Aliphatic	1.0
C	Aromatic	1.0
C	Olefinic	0.95
C	Acetylenic	1.30
C	Carbonyl	0
C	Carboxyl	0
C	Nitrile	0.3
O	Ether	-1.0
O	Primary alcohol	-0.5
O	Secondary alcohol	-0.75
O	Tertiary alcohol	-0.25
N	In amines	Similar to O in alcohols
Cl	On olefinic C	-0.05
Cl	Two or more on aliphatic C	-0.12 per Cl

a conventional FID by locating the ion collection electrodes several centimeters downstream of an oxygen-rich flame. The HAFID and RFID provide favorable detection limits (pg/s) and selectivity ratios but are considered curiosities probably because of their limited application range and competition from other element-selective detectors, such as the atomic emission detector (section 3.10.3.3). The O-FID is an oxygen-selective detector using a postcolumn reaction system to convert oxygen into methane, which is then detected by a standard FID [34]. The reaction system consists of a cracking reactor (a metal tube heated to $> 850^{\circ}\text{C}$), which converts oxygen atoms into carbon monoxide and a separate methanizer (short Al_2O_3 PLOT capillary column impregnated with nickel catalyst) inserted in the FID jet, which converts the carbon monoxide to methane. Hydrocarbon compounds are converted to carbon and hydrogen in the cracking reactor and do not interfere in the detection of oxygen-containing compounds. The main use of the O-FID is the selective determination of oxygen-containing additives in hydrocarbon fuels.

3.10.1.2 Thermionic Ionization Detector

Modern thermionic ionization detectors evolved out of earlier studies of alkali-metal-doped flame ionization detectors [253]. Adding an alkali metal salt to a flame enhanced the response of the detector to compounds containing certain elements, such as N, P, S, B, as well as some metals (e.g. Sb, As, Sn, Pb). In its early versions, however, the detector response was unreliable and critically dependent on experimental parameters. Recent studies involving the continuous introduction of alkali metal salt solutions or aerosols into the flame demonstrated more reliable performance but have not been taken up [268,269].

All modern thermionic ionization detectors (TID) employ a solid surface, composed of a ceramic or glass matrix doped with an alkali metal salt in the form of a bead or cylinder, molded onto an electrical heater wire as the thermionic source [254,270,271].

The current applied to the electrical heater controls the source temperature, which is typically set to a value between 400-800°C. The column carrier gas is combined with hydrogen at the detector base and flows through a jet where it is mixed with air. The flow of detector gases is insufficient to establish a flame, but sufficient to maintain a plasma localized around the thermionic source. Hydrogen and air flow rates are typically 2-6 and 60-200 ml/min, respectively, about 5 fold lower than those employed in a typical FID. The thermionic source is located immediately above the jet tip and the cylindrical collector electrode either surrounds the source or is located immediately above it, depending on the detector design. A voltage set between the collector electrode and jet tip allows collection of (usually) negative ions and the ion current is measured by an electrometer.

The dilute mixture of hydrogen in air forms a plasma (or boundary layer) localized at the surface of the hot thermionic source. The source is responsible for dissociation of hydrogen molecules into reactive hydrogen radicals and formation of a reactive chemical environment containing hydrogen, oxygen and hydroxyl radicals, water and the original detector gases. Compounds entering the hot reactive boundary layer are efficiently decomposed into fragments. The detailed mechanism whereby these decomposition products become selectively ionized is less clear.

Two different ionization mechanisms have been proposed to explain the element-selective response of the TID to nitrogen- and phosphorous-containing compounds [254,259,271-275]. These mechanisms differ principally in whether the interaction between the alkali metal atoms and organic fragments occurs as a homogeneous reaction in the gas phase or is purely a surface phenomenon. According to the gas-phase ionization theory, alkali metal atoms are vaporized from the hot source after acceptance of an electron from the heating wire or plasma. While in the boundary layer, the alkali metal atoms are excited and ionized by collision with plasma particles. The negatively charged source rapidly recaptures the positively charged metal ions. This cyclic process results in a steady state population of metal atoms in the boundary layer. If a process that results in ionization of metal atoms disturbs this equilibrium then more metal atoms will leave the source to restore the equilibrium accompanied by an increase in the ion current. The selectivity of the detector results from the fact that only those radicals with electron affinities equal to or greater than the ionization potential of the metal atoms will contribute to the ion current. Among the many fragments generated by the decomposition of organic compounds in the plasma, only the CN^\bullet , PO^\bullet , and PO_2^\bullet radicals meet this criterion for the alkali metals. Spectroscopic and mass spectrometric experiments, however, failed to provide any corroborative evidence for the gas-phase ionization mechanism, which has lost ground in recent years to the surface ionization theory. The surface ionization model assumes that the principal role of the alkali metal in the source is to lower the work function of the surface (i.e. the amount of energy required to emit a unit of electrical charge from the surface). Electronegative decomposition products from phosphorus- or nitrogen-containing compounds are then selectively ionized by extracting an electron from the surface of the thermionic source. These negative ions are responsible for the increase in observed ion current measured

at the collector electrode. To account for the influence of experimental variables on the detector response it is further assumed that a combination of the heat from the source and the reaction of sample molecules within the boundary layer are responsible for sample decomposition. The active decomposition products must be similar and largely independent of molecular structure for nitrogen- and phosphorous-containing compounds to account for the limited range of response factors.

The response of the detector to different elements depends on the electronic work function of the thermionic surface, the chemical composition of the gas environment immediately surrounding the thermionic surface, and the temperature of the thermionic surface [254,270,276]. With only nitrogen as the plasma gas very specific ionization of compounds containing functional groups of high electron affinity, such as nitro, halogen, and thiol groups, etc., can be obtained. This requires a thermionic source with a low work function (high cesium content) but the response is very structure dependent. With air or oxygen as the plasma gas and a relatively low source temperature (400 to 500°C) enhanced selectivity towards halogenated compounds is obtained. Using air mixed with a low flow of hydrogen as the plasma gases provides selectivity towards nitrogen- and phosphorus-containing compounds, as discussed above.

Both the selectivity and sensitivity of the detector are dependent on experimental variables, principally the source heating current, source location, jet potential, air and hydrogen flow rates and choice of carrier gas [254,277-279]. All versions of the thermionic detector exhibit loss of sensitivity and selectivity with use necessitating periodic source replacement and frequent checks on calibration. Peak tailing is sometimes observed because sample decomposition products remain temporarily adsorbed on the thermionic source. Operation at a higher temperature or with a higher hydrogen flow rate may correct this problem. Injection of excess silylation reagents or halogenated solvents seems to accelerate source deterioration or disturb detector stability. Amides and nitro-containing compounds tend to produce lower responses than other nitrogen-containing compounds.

Although, the detector response mechanism is poorly understood, the working limits of commercially available detectors are fairly well characterized and the detector is not particularly difficult to use. The minimum detectable amount of nitrogen is about 5×10^{-14} to 2×10^{-13} g N/s and for phosphorus about 1×10^{-14} to 2×10^{-13} g P/s. The selectivity against carbon is about 10^3 to 10^5 g C/g N and 10^4 to 5×10^5 g C/g P. Discrimination against phosphorus in the nitrogen mode is poor at 0.1 to 0.5 g P/g N. The linear response range is about 10^4 to 10^5 . The TID is widely used in environmental and biomedical research for determining pesticides residues and drugs as well as for obtaining element-selective profiles, where its high sensitivity and selectivity are useful in minimizing sample preparation requirements. The TID is also known as the nitrogen-phosphorus detector (NPD).

3.10.1.3 Photoionization Detector

Ionization is a possibility when a molecule absorbs a photon of energy close to or greater than its ionization potential. For organic compounds this requires photons in

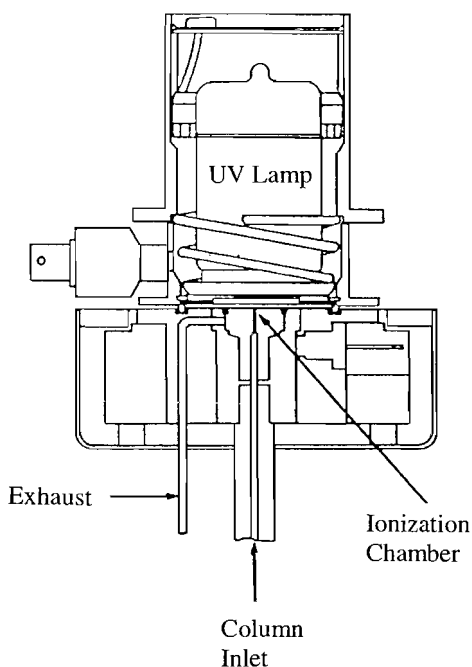


Figure 3.20. Cross-sectional view of a photoionization detector.

the far ultraviolet of about 5-20 eV. Early photoionization detectors (PID) operated at low pressures to maximize the photon intensity but this was not very convenient. The first commercially successful PID detectors employed a design that physically separated the photon source from the ionization chamber allowing independent optimization of photon production and ion collection [254,280,281]. Due to the low efficiency of photoionization ($< 0.1\%$) the detector is classified as nondestructive. Since no combustion or support gases, beyond makeup gas for some applications, are required the PID can be used in environments where combustion gases are considered hazardous or in portable instruments, where the additional weight of several gas bottles is undesirable. The PID is a concentration sensitive detector and its response flow rate dependent. The ionization efficiency is compound dependent and individual calibration curves are required for each substance to be quantified.

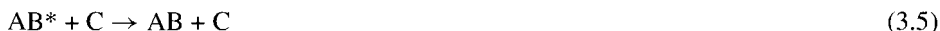
A cross-sectional view of a typical photoionization detector is shown in Figure 3.20. The photon source is a compact discharge lamp, containing an inert gas or gas mixture at low pressure, that emits monochromatic light of a specific energy, depending on the choice of fill gases and window material. Sources of different nominal energies (8.3, 9.5, 10.2, 10.9 and 11.7 eV) provide for the possibility of selective ionization of organic compounds with the 10.2 eV source used for general applications. An optically transparent window made of a metal fluoride separates the discharge compartment from

the ionization chamber. The column effluent passes through the thermostatted ionization chamber and between two electrodes, positioned at opposite ends of the chamber. It is important that the collector electrode is shielded from the photon flux from the source to minimize background noise. Detectors with ionization chamber volumes of 40 and 175 μl are available for use with open tubular and packed columns, respectively. An electric field is applied between the electrodes to collect the ions formed (or electrons, if preferred) and the current amplified by an electrometer. A pulsed discharge photoionization detector was recently described using an open source design [282-284]. Photons are generated by an atmospheric pressure helium discharge that can be doped with other inert gases to provide a range of photon energies. The detector is constructed from a quartz tube divided into two zones with electrodes located at the end flanges and middle section. A high voltage pulse is used to initiate the discharge. Since the pulse discharge PID does not require a barrier between the source and ionization chamber it can be operated at lower wavelengths than the conventional PID design functioning as a near universal detector.

The selectivity of the PID results from the relationship between the photon energy and the compound ionization potential. The discharge lamps are indicated by a single energy level but may emit several photons of different energy [254,285,286]. The 8.3 eV lamp is filled with xenon and emits photons of 8.44 eV. It is used to selectively ionize compounds with a low ionization potential, such as polycyclic aromatic compounds, with minimum interference from other compounds. The 9.5 eV lamp is filled with xenon and emits photons predominantly of 8.44 eV (97.6%), 9.57 eV (2.1%) and 10.40 eV (0.18%). It is used primarily to determine simple aromatic compounds in an alkane matrix, mercaptans in hydrogen sulfide and amines in ammonia. The 10.2 eV lamp is filled with krypton and emits photons of 10.03 eV (82.9%) and 10.64 eV (17.1%). It is a general-purpose source with a high photon flux that ionizes most compounds except for low molecular weight compounds such as the permanent gases, C_1 - C_4 alkanes, methanol, acetonitrile and chloromethanes. The 11.7 eV lamp is filled with argon and emits photons of 11.62 eV (71.8%) and 11.82 eV (26.2%). It is used sparingly to detect low molecular weight compounds with high ionization potentials (e.g. formaldehyde, chloromethanes, ethane, acetylene, etc). Most lamps have a working life >5000 h with the exception of the 11.7 eV lamp. Its lifetime is limited due to window (lithium fluoride) absorption of photons forming colored centers (solarization) that attenuate the photon intensity. Heating the window material to above 125°C further reduces the photon flux. There is no PID lamp comparable to the helium pulsed discharge PID detector (13.5 to 17.8 eV) since there are no suitable window materials for this region of the spectrum.

Absorption of a photon by a molecule AB is accompanied by a number of competing processes in addition to ionization involving carrier gas molecules C and electron-capturing impurities EC described by the following equations [287-289].





Eq. (3.2) represents capture of a photon by AB resulting in ionization and a detector signal. Eqs. (3.3)-(3.5) represent competing reactions that reduce the detector signal and should be minimized. These reactions include the loss of product ions by recombination (Eq. 3.3) and neutralization by reaction with an electron-capturing impurity (Eq. 3.4). Eq. (3.5) represents collisional de-excitation by a carrier gas molecule. The choice of carrier gas can influence the detector response through the collision processes represented by Eqs. (3.3) and (3.5), as a source of electron-capturing impurities (e.g. O₂), and by its ability to influence the mobility of ions within the detector [287]. When competing reactions are minimized, the response of the detector can be described by Eq. (3.6) [288].

$$i = I F \eta \sigma N L [\text{AB}] \quad (3.6)$$

where i is the detector ion current, I the initial photon flux, F the Faraday constant, η the photoionization efficiency, σ the absorption cross-section, N Avogadro's number, L the path length and $[\text{AB}]$ the concentration of an ionizable substance. Thus, for a particular detector and source, the PID signal is proportional to the ionization yield, absorption cross-section and molar concentration of the analyte. The product ($\eta\sigma$) is the photoionization cross-section, which expresses both the probability that a molecule will absorb a photon and the probability that the excited state will ionize. The calculation of photoionization cross-sections is complex but intuitively a direct dependence of the photoionization cross-section on the photon energy and the ionization potential of the molecule is expected. In practice, a fraction of the molecules with ionization potentials up to approximately 0.4 eV above the photon energy will be ionized, as some of these molecules will exist in vibrationally excited states. Ionization potentials can be calculated from molecular orbital theory [282,290] and many values for common compounds are tabulated [254,285,291], which aids in identifying a suitable photon source for the detection of a particular compound

The PID is nondestructive, relatively inexpensive, of rugged construction and easy to operate. The linear range is approximately 10^7 . For favorable compounds the PID is 5 to 50 times more sensitive than the FID [280,286]. In other cases it may not respond at all or respond poorly determined by the ionization potential of the compound and the photon energy and flux. On an individual compound basis relative detector response factors vary over a wide range allowing the PID to be used as a selective detector for some applications. Major applications of the PID are the analysis of volatile organic compounds from environmental samples and in field-portable gas chromatographs [292].

3.10.1.4 Electron-Capture Detector

The structure-selective, electron-capture detector (ECD) is the second most widely used ionization detector [254,293-295]. It owes its popularity to its unsurpassed sensitivity to a wide range of environmentally important and biologically active compounds. Examples of general applications include the determination of pesticides and industrial chemicals in the environment, assessment of the fate of ozone-depleting chemicals in the upper atmosphere and the determination of drugs and hormones in biological fluids.

The ECD is available in two forms that differ in the method used to generate the equilibrium concentration of thermal electrons responsible for the operating characteristics of the detector. From inception high-energy beta electrons generated by the decay of a radioisotope source have been used as the primary source of ionizing radiation. These particles produce a large number of secondary electrons through multiple collisions with carrier gas molecules forming a plasma of thermal electrons (mean energies 0.02 to 0.05 eV), radicals and positive ions. Radioisotope-based detectors require periodic wipe tests to ensure safety as well as compliance with regulations concerning storage, use and transport of radioactive materials. Regulatory constraints provided the impetus for the development of a non-radioactive detector. The non-radioactive ECD uses high-energy photons from a pulsed discharge in pure helium to photoionize a support gas added downstream of the discharge. A plasma of thermal electrons and positive ions is formed in the ionization chamber with properties similar to the plasma generated by the radioactive ECD. Application of a fixed or pulsed potential to the ionization chamber allows the thermal electrons to be collected and a standing (or baseline) current established. When an electron-capturing compound enters the ionization chamber thermal electrons are removed by formation of negative ions. The increased rate of neutralization of these ions by positive ions, or their reduced drift velocity during collection of the thermal electrons, is responsible for the detector signal. In contrast to the other ionization detectors a decrease in the detector standing current proportional to solute concentration is measured rather than an increase in the number of ions or electrons produced.

The ideal source of ionizing radiation would produce a small number of ion pairs per disintegration to minimize the fluctuations in the ion current. At the same time, the total ion pair formation should be large so that the resulting electron current during the passage of an electron-capturing substance can be measured conveniently without introducing other sources of noise. The best compromise among these demands appears to be low energy beta-emitting radioisotopes (minimum number of ion pairs per particle) at relatively high specific activity (maximum total ion pair formation). Either ^{63}Ni or ^3H radioisotope sources supported by a metal foil are used in commercial detectors. Of the two types, tritium would be preferred due to its lower energy particle emission (0.018 MeV) compared to ^{63}Ni (0.067 MeV) and the fact that tritium foils with higher specific activity are less expensive to manufacture. The principal advantage of ^{63}Ni sources is their high temperature stability (to 400°C). The majority of radioactive ECD detectors, however, use ^{63}Ni for its operational and practical convenience.

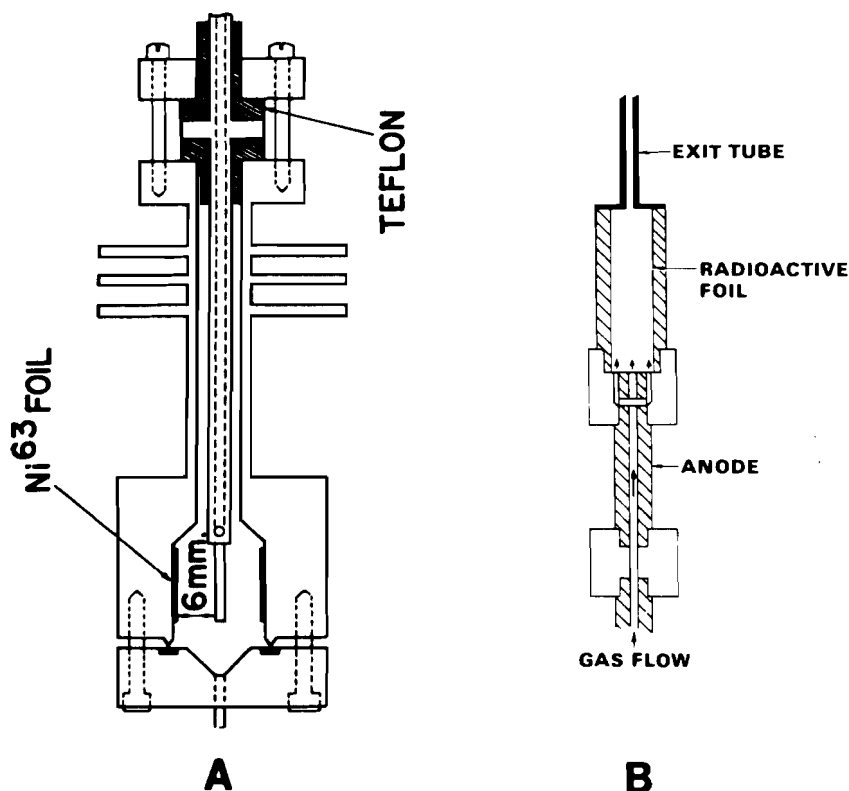


Figure 3.21. Cross-sectional view of the coaxial cylinder (A) and asymmetric [displaced coaxial cylinder] (B) configurations for the electron-capture detector.

Over the years many designs have been proposed for the radioactive ECD. The majority of detectors in use today are related in one way or another to either the coaxial cylinder or asymmetric configurations shown in Figure 3.21 [293,296-301]. The low specific activity of the ^{63}Ni source requires the use of a larger source area to obtain the desired background current. This is more easily accommodated in the coaxial cylinder design. In this case, the penetration length of the beta particles establishes the minimum distance between the source, which surrounds the centrally located anode, and the anode. This distance should be large enough to ensure that all beta particles are deactivated by collisions and converted to thermal energies without colliding with the anode. In the asymmetric design, locating the anode entirely upstream from the ionized gas volume minimizes the collection of long-range beta particles. In addition, the direction of gas flow minimizes diffusion and convection of electrons to the anode. However, the free electrons are sufficiently mobile that a modest pulse voltage is adequate to cause the electrons to move against the gas flow for collection.

Miniaturization of the ionization chamber is important for use with open tubular columns [254,294]. The effective minimum volume of the asymmetric configuration is about 100-400 μl , which is still too large to eliminate extracolumn band broadening completely with open tubular columns [300,301]. The coaxial cylinder design has a larger ionization chamber volume, sometimes 2 - 4 ml, suitable for packed column separations only. The effective detector dead volume can be reduced by adding makeup gas at the end of the column to preserve column efficiency at the expense of a reduced detector response due to sample dilution. Pure argon and helium are unsuitable makeup gases as they readily form metastable species, which can transfer their excitation energy by collision with solute vapors, resulting in undesirable ionization effects (Penning reaction). The addition of 5 to 10 percent of methane to argon removes these ions by deactivating collisions as quickly as they are formed. Oxygen-free nitrogen is the most common carrier gases used with packed columns. Hydrogen or helium are usually used as carrier gases with open tubular columns to maximize column efficiency and minimize separation time while argon-methane or nitrogen are used as makeup gases. Oxygen and water vapor traps should be used to purify all gases.

Space charge, contact potential effects and other non-electron-capturing reactions, render the collection of thermal electrons with a constant voltage unreliable [293]. Virtually all radioisotope source detectors use pulse sampling techniques to collect the thermal electrons. The potential is applied to the ionization chamber as a square-wave pulse of sufficient height and width to collect all the thermal electrons and with sufficient time between pulses for the concentration of thermal electrons to be replenished by the ionizing beta radiation, and for their energy to reach thermal equilibrium. The signal from an ECD, operated with a long pulse period, can be described by Eq. (3.7)

$$(I_b - I_e)/I_e = K [AB] \quad (3.7)$$

where I_b is the detector standing current, I_e the detector current measured at the maximum peak height for an electron-capturing compound of concentration $[AB]$, and K the electron-capture coefficient. By analog conversion the detector signal can be linearized over about four orders of magnitude [302].

Most ECD detectors are designed for use with a modified version of the pulsed-sampling technique termed the variable frequency constant-current mode [303]. In this case, the pulse frequency is varied throughout the separation to maintain the cell current at a fixed reference value. The detector signal is a voltage proportional to the pulse frequency. The two principal advantages of this method are an increased linear response range of 10^4 to 10^5 and reduced detector disturbance from column contamination. For a few compounds with ultrafast electron attachment rate constants (e.g. CCl_4 , SF_6 , CFCl_3 , CH_3I and certain polychlorinated biphenyl congeners) the detector response is non-linear [298,304-308]. For compounds with slower rate constants the detector response is normally linear over the full operating range. This group includes most of the compounds with moderately strong electrophores that make up the majority of compounds determined with the ECD.

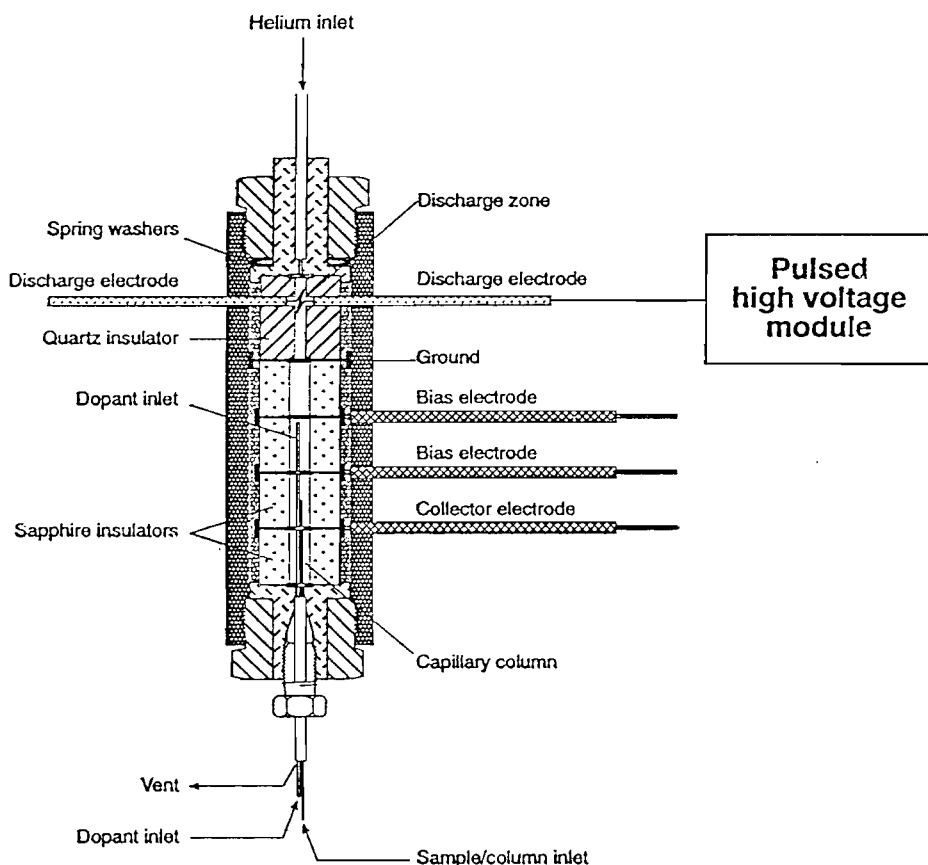


Figure 3.22. Cross-sectional view of a pulsed discharge electron-capture detector operating in the constant current mode. (From ref. [308]; ©American Chemical Society).

A cross-sectional view of the pulsed discharge electron-capture detector (PDECD) is shown in Figure 3.22 [295,308,309]. A pulsed discharge in pure helium is used to generate high-energy photons from diatomic helium excimers (13.5-17.5 eV) to ionize a dopant gas, usually methane, added down stream of the discharge. The helium discharge gas (30-40 ml/min) is introduced at the top of the detector and flows through the discharge and into the ionization region. The flow is strong enough to minimize diffusion of the dopant gas and column effluent into the discharge region. The dopant gas (methane) is introduced between the upper two electrodes where there is a slight difference in potential, to assist the migration of electrons into the electron-capture region between the two lower electrodes. The column effluent is introduced through a fitting at the detector base and its end positioned close to the middle electrode in the upper portion of the electron-capture region. The detector

Table 3.4

Relative response of the electron-capture detector to various organic compounds

General organic compounds	Relative response	Fluorocarbon compounds	Relative response
Benzene	0.06	CF ₃ CF ₂ CF ₃	1.0
Acetone	0.50	CF ₃ Cl	3.3
Di-n-butyl ether	0.60	CF ₂ =CFCl	1.0 x 10 ²
Methylbutyrate	0.90	CF ₃ CF ₂ Cl	1.7 x 10 ²
1-Butanol	1.00	CF ₂ =CCl ₂	6.7 x 10 ²
1-Chlorobutane	1.00	CF ₂ Cl ₂	3.0 x 10 ⁴
1,4-Dichlorobutane	15.00	CHCl ₃	3.3 x 10 ⁴
Chlorobenzene	75.00	CHCl=CCl ₂	6.7 x 10 ⁴
1,1-Dichlorobutane	1.1 x 10 ²	CF ₃ Br	8.7 x 10 ⁴
1-Bromobutane	2.8 x 10 ²	CF ₂ ClCFCl ₂	1.6 x 10 ⁵
Bromobenzene	4.5 x 10 ²	CF ₃ CHClBr	4.0 x 10 ⁵
Chloroform	6.0 x 10 ⁴	CF ₃ CF ₂ CF ₂ I	6.0 x 10 ⁵
1-Iodobutane	9.0 x 10 ⁴	CF ₂ BrCF ₂ Br	7.7 x 10 ⁵
Carbon tetrachloride	4.0 x 10 ⁵	CFCl ₃	1.2 x 10 ⁶

has an effective volume of about 55 μ l and is suitable for use with open tubular columns.

Early versions of this detector operated in the fixed frequency pulse mode but later versions employ the constant-current mode [308]. The variable frequency constant-current mode used in the radioactive ECD is not applicable to the PDECD. The PDECD uses a variable dc bias potential to maintain a constant direct current. The increase in bias voltage is proportional to the concentration of compound entering the detector and provides the detector signal. Operation in the constant current mode extends the linear operating range of the detector and simplifies its operation. In general, the response mechanism of the PDECD is identical to the radioactive ECD. The sensitivity of the PDECD is equal to or better than that of the radioactive ECD with a linear response range of 10⁴-10⁵.

The ECD is a structure-selective detector with a response range that covers about seven orders of magnitude, Table 3.4 [293,310]. The response is greatest to compounds containing halogen atoms or nitro groups, to organometallic compounds, and to conjugated electrophores. This latter group is the least well defined, and is comprised of compounds containing two or more weakly electron-capturing groups, connected by some specific bridge that promotes a synergistic interaction between the two weak electron-capturing groups [311]. Examples of conjugated systems with a high detector response include conjugated carbonyl compounds (benzophenones, quinones, phthalate esters, coumarins), some polycyclic aromatic hydrocarbons, some sulfonamides and certain steroids. The response of the ECD to halogen-containing compounds decreases in the order I > Br > Cl » F and increases synergistically with multiple substitution on the same carbon atom. The response of the ECD to haloaromatic and nitroaromatic compounds shows similar trends to the alkyl compounds. The position of electronegative functional groups (ortho, meta, para)

has a measurable influence on the detector response for aromatic compounds but is less dramatic than the response variation due to the number and type of individual substituents. The law of diminishing returns applies to all organic compounds; the introduction of the first few electronegative substituents has a large impact on detector sensitivity, but further substitution has less influence. If sufficient numbers of electronegative groups are introduced, as for example in polychlorinated biphenyls, the response limit for the detector is reached.

Nearly all that is known about the structure-selective response of the ECD is based on empirical observations. Clearly, the ability to correlate the ECD response to fundamental molecular properties would be useful. Chen and Wentworth have shown that the information required for this purpose is the electron affinity of the molecule, the rate constant for the electron attachment reaction and its activation energy, and the rate constant for the ionic recombination reaction [293,312]. In general, the direct calculation of detector response factors has rarely been carried out, since the electron affinities and rate constants for most compounds of interest are unknown and difficult to determine.

General models of the electron-capture process are based on the kinetic model of Wentworth and co-workers [254,293,295,298,313-315]. The ionization chamber is treated as a homogeneous reactor into which electrons are continuously introduced at a constant rate and electron-capturing solutes are added at a variable rate in a constant flow of carrier gas. The major consumption of electrons is via electron capture and recombination with positive ions. The model can be expanded to allow for the presence of electron-capturing contaminants and the formation of excited state negative ions. The kinetic model provides a reasonable explanation of the influence of pulse sampling conditions and temperature on the detector response, but exactly calculated solutions are rare. Again, this is because the necessary rate constants are usually unavailable, and the identity and relative concentration of all species present in the detector are uncertain. The principal reactions can be summarized as follows:

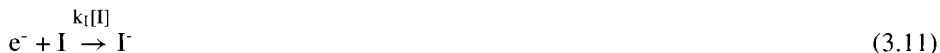
(i) Reaction of sample molecules (AB) with thermal electrons to form negative ions by either instantaneous dissociation (Eq. 3.8) with a rate constant k_{12} or the formation of a stable negative ion by resonance capture with a rate constant k_1 , which can subsequently dissociate with a rate constant k_2 (dissociative capture) or detach with a rate constant k_{-1} .



(ii) The overall loss of electrons by recombination with positive ions (P^+)



(iii) Loss of electrons due to capture by carrier gas impurities of concentration [I]



(iii) Loss of any of the negative ions by combination with positive ions



For Eq. (3.10) and Eq. (3.12) the bimolecular rate constants can be replaced with pseudo unimolecular rate constants within the limits of either fractional electron capture or constant positive ion concentration. All the above reactions take place on a time scale that is fast relative to the time required for transport through the detector. Under steady state conditions the electron capture coefficient K (see Eq. 3.7) is given by

$$K = (k_{I2} + [k_1 \{k_2 + k_N\} / \{k_{-1} + k_2 + k_N\}]) / (k_D + k_I[I]) \quad (3.13)$$

For compounds that capture electrons predominantly by a single reaction pathway some rate constants are negligible and Eq. (3.13) can be simplified. The model can be extended to include stable excited states that are involved in the electron-capture process for a minority of compounds [315]. The detector response is also a function of the cleanliness of the detector represented by the contribution of $k_I[I]$ to the electron capture coefficient.

The influence of temperature on the detector response is of considerable practical significance (the response can vary by as much as 10^2 - 10^3 for a 100°C change in detector temperature). It is also a useful tool for the calculation of electron affinities, and for elucidating mechanistic details of the electron-capture process [293,315-319]. This strong temperature dependence can be derived directly from the kinetic model of the detector response. Resonance electron-capture occurs with the formation of a stable molecular ion (Eq. 3.9 $k_1 \gg k_2$). Alternatively, dissociative electron capture requires formation of a molecular ion in a sufficiently excited state that instantaneous (Eq. 3.8) or rapid (Eq. 3.9 $k_2 \gg k_1$) bond breaking occurs accompanied by elimination of a negative ion. Resonance electron capture is generally associated with conjugated or delocalized structures with low energy molecular orbitals that can accept an electron to form a stable negative ion. Dissociative electron capture is commonly observed for halogen-containing compounds and is promoted by the relatively low carbon-halogen bond energies and the high electron affinity of the halogen atoms. The favored mechanism depends on the juxtaposition of the potential energy curves for the neutral molecule and the negative ion. In the case of resonance electron capture the negative molecular ion is more stable than the neutral molecule, whereas in the dissociative case the potential energy curve for the negative ion crosses that of the neutral molecule at a level corresponding to a vibrationally excited state. An increase in detector temperature favors the populating of vibrationally excited states and thus the mechanism represented

by Eq. (3.8). Conversely, resonance electron capture may be destroyed if the rate constant for thermal detachment is too fast, and a greater detector response is usually observed at low detector temperatures. The two mechanisms can be distinguished from a Arrhenius-type plot of $\ln KT^{3/2}$ (or since the electron capture coefficient is probably unknown, a plot of $\ln AT^{3/2}$, where A is the peak area for a constant sample size) against $1000/T$, where T is the detector temperature. In most cases the plots will be linear with a negative slope characteristic of dissociative electron capture and a positive slope characteristic of resonance electron capture. For practical work, the optimum detector temperature for maximum response is usually either the maximum temperature for dissociative electron-capture compounds, or the lowest practical operating temperature for resonance electron-capture compounds. Nonlinear and structured plots are observed for a small number of compounds resulting from significant changes in the relative reaction rate constants over a small temperature range, or when stable excited state ions are formed [319].

The ECD is a concentration dependent detector and its response depends on the flow rate and purity of the carrier gas, as well as specific detector design and operating characteristics (including detector temperature), and the contribution of background contamination from the column and chromatographic system. For quantification response data are usually transposed into concentrations by calibration and, since response factors cover a wide range for electron-capturing compounds, each substance must have an individual calibration curve. For compounds that are virtually completely ionized it has been suggested that the ECD could be operated in an absolute, or coulometric mode. In this case, the time integral of the number of electrons captured during the passage of a chromatographic peak is equal, via Faraday's law, to the number molecules ionized [320-322]. This approach would be particularly useful for the quantitative analysis of certain compounds at concentration levels where accurate calibration standards are difficult to prepare. Practically, it has proven difficult to construct devices that function reliably as coulometric detectors. The main reason is probably the complex and changing chemistry that occurs in the detector ionization chamber.

The intentional addition of dopants such as oxygen, nitrous oxide, or ethyl chloride to the makeup gas of a standard ECD can provide a significant increase in detector response to certain otherwise weakly electron-capturing compounds [254,293]. This technique was called chemically sensitized electron capture detection. When operated in this mode the ECD functions as an ion-molecule reactor in which the detector response is determined by the rate of an ion-molecule reaction rather than the usual electron capture reaction. Because the dopants are present in relatively high concentration compared with the analytes, electron capture occurs virtually exclusively with the dopant, forming O_2^- ions in the case of oxygen and O^- ions in the case of nitrous oxide, as the reactant species. Virtually all organic compounds react with the O^- ions producing a non-selective response. Oxygen provides a selective response for compounds that react rapidly with O_2^- ions but not with electrons (e.g. alkyl halides, polycyclic aromatic compounds). Ethyl chloride is used to enhance the response of compounds that capture

electrons by resonance capture (e.g. polycyclic aromatic compounds) but have a poor response because the electron detachment reaction is too fast. In this case the unstable negative molecular ion is replaced by the stable chloride ion. In favorable cases operating the ECD in the chemically sensitized mode can enhance the detector response by 10^3 to 10^4 but only a few demonstration experiments have been performed and no general applications have resulted from these studies.

3.10.1.5 Helium Ionization Detector

The helium ionization detector (HID) is a universal and ultra sensitive detector used primarily for the analysis of permanent gases and some volatile organic compounds that have a poor response to the FID and are present in too low a concentration for detection with a thermal conductivity detector [254]. Many aspects of the design of the HID are similar to those of the ECD with which it shares a common parentage. The two common versions of the HID are based on either a radioactive source [254,323] or a pulsed discharge [309,324-326] to generate metastable helium species thought to be responsible for sample ionization by collision. The HID has a reputation as a difficult detector to use and must be operated under stringent conditions with respect to contamination from carrier gases impurities and the ingress of air or column bleed. Because applications have remained focused on inorganic gases and simple volatile organic compounds gas-solid columns are often used for separations to minimize contamination problems and drifting baselines with poorly conditioned or contaminated liquid stationary phases.

The radioactive HID consists of a 100-200 μ l thermostatted ionization chamber with a parallel plate or coaxial cylinder electrode configuration. High temperature operation is uncommon so tritium sources of a high specific activity (0.25-1.0 Ci) are used to maximize sensitivity. The detector current is measured by applying a constant or pulsed voltage to the electrodes in either the saturation or multiplication region. Pulsed sampling provides improved baseline stability but lower sensitivity than the constant potential mode. Likewise, voltages around 550 V (in the multiplication region) provide a greater response accompanied by increased background noise. The detector is usually more sensitive if less stable and more disturbed by contamination when operated in this region. The pulsed discharge HID is similar to the pulsed discharge ECD, Figure 3.22, and requires only simple and unintrusive modifications to operate in either mode. In the pulsed discharge HID mode the column exit is relocated to the discharge region, the dopant gas supply is disconnected, and the potentials on the electrodes and position of the electrometer connections are changed. The sensitivity and linear range of the pulsed discharge HID depends on the power of the pulsed discharge, the pulse frequency, the voltage applied to repel electrons to the collector electrode and the helium flow rate through the discharge region. Either detector configuration is capable of measuring parts-per-billion concentrations ($1-20 \times 10^{-12}$ g on column) of compounds with a linear response range of about 10^4 to 10^5 .

3.10.2 Bulk Physical Property Detectors

The bulk physical property detectors respond to some difference in a carrier gas property due to the presence of the analyte. Usually, a large signal for the characteristic carrier gas property is desired to provide a reasonable working range, but for low concentrations of analyte the detector signal corresponds to a very small change in a large signal, and is noise limited. The sensitivity of the bulk physical property detectors tends to be low compared to ionization detectors. The most important of the bulk physical property detectors are the thermal conductivity detector, gas density balance, and the ultrasonic detector. Of these, only the thermal conductivity detector is widely used.

3.10.2.1 Thermal Conductivity Detector

The thermal conductivity detector (TCD) is a universal, non-destructive, concentration-sensitive detector that responds to the difference in thermal conductivity of the pure carrier gas and the carrier gas containing analyte. The TCD is generally used to detect permanent gases, light hydrocarbons and compounds that respond only poorly to the flame ionization detector (FID). In many applications it has been replaced by the FID, which is more sensitive (100- to 1000-fold), has a greater linear response range, and provides a more reliable signal for quantification. Detection limits for the TCD usually fall into the range of 10^{-6} to 10^{-8} g per peak with a response that is linear over about four orders of magnitude. If lower detection limits are required for compounds with a poor response to the FID then the helium ionization detector (HID) is a suitable alternative.

In a typical TCD, the carrier gas flows through a heated thermostatted cavity that contains the sensing element, either a heated metal wire or thermistor. With pure carrier gas flowing through the cavity the heat loss from the sensor is a function of the temperature difference between the sensor and cavity and the thermal conductivity of the carrier gas. When carrier gas containing an analyte enters the cavity, there is a change in the thermal conductivity of the carrier gas mixture and a resultant change in the temperature of the sensor. The sensor may be operated in a constant current, constant voltage, or constant mean temperature mode as part of a Wheatstone bridge network. A temperature change in the sensor results in an out-of-balance signal proportional to the concentration of analyte in the sensor cavity.

The TCD has appeared in several different designs, some of which have advantages for particular applications [327,328]. They usually represent some variation of the three basic geometries: the flow-through, semi-diffusion and diffusion cells. In the flow-through cell, carrier gas passes over the sensor and in the diffusion cell the sensor is located in a recess into which a portion of the carrier gas stream enters by diffusion. The diffusion and semi-diffusion cells have a slow response, a relatively large detector volume and are relatively insensitive; they are used mainly for packed column analytical and preparative chromatography. Flow-through cells with volumes of 1 to 100 μ l are easily fabricated for use with open tubular columns [329,330]. In a different approach, flow modulation is used to switch the carrier gas between two channels, one of which contains a single filament. Every 100 milliseconds a switching

valve fills the filament channel alternately with carrier gas and column effluent. No reference column is required and, with an effective detector volume of 3.5 μl , it can be used with even narrow bore open tubular columns. Digital data processing and more reliable temperature compensation provide greater sensitivity and stability than conventional detector designs. Thermal conductivity detectors with nanoliter volumes have been fabricated using micromachining techniques for the development of a chip-based gas chromatograph, but have not been widely used [331].

Temperature gradients within the detector cavity result in poor detector performance. At the detection limit temperature changes as small as 10^{-5}°C are encountered, and it is not possible to thermostat a cell to provide the necessary absolute thermal stability to measure these small temperature changes. The sensing element must be centrally located within a detector body having a large thermal mass of controlled temperature ($\pm 0.01^{\circ}\text{C}$). A difference signal must be used by incorporating a matched reference cell in the same environment as the sample cell or by using flow modulation if a single filament design is used. Some designs use two sampling and two reference cells to improve stability.

Carrier gases of low molecular weight and high thermal conductivity (e.g. H_2 and He) are required to maximize the detector response and to maintain a large linear response range. Heavier carrier gases such as nitrogen, as well as influencing sensitivity and linearity, may give rise to negative or split top sample peaks. Theoretical models have been advanced to explain the response characteristics of the detector under different operating conditions [332,333]. These models take account of the effects of conduction, convection and radiation on the loss of heat from the sensor, but do not lead to any simple mathematical expression to describe its operation. A number of response factor compilations for different carrier gases are available, usually expressed on a weight or molar response basis relative to benzene [252,328,334]. These values are generally sufficiently accurate for estimating sample concentrations. For more accurate results it is necessary to calibrate the detector for each substance individually. Alternatively, an internal standard can be used to determine accurate response ratios that can be used for quantitative analysis.

3.10.3 Optical Detectors

Absorbance detectors are little used in gas chromatography in contrast to the majority of separation techniques using a liquid mobile phase [335,336]. A contributing factor is that the vast majority of absorption maxima of diagnostic interest occur at wavelengths shorter than 195 nm. Spectral recording over the wavelength range 168–330 nm is possible using a temperature controlled lightpipe (volume = 170 μl) interfaced to a photodiode array spectrometer. Library searchable UV spectra can be obtained from sub-nanogram amounts of organic compounds that possess reasonable spectral features. The position and shape of spectral bands are temperature dependent, setting some constraints on their use for identification purposes. In general UV spectra are less informing but complementary to mass and infrared spectral data, but so far have not been widely used for identification purposes.

The use of flames as atom reservoirs for the spectroscopic determination of elements is a well-established technique that is particularly valuable for metal analysis. The principal emission or absorption lines for most non-metallic, element-containing compounds, which account for the majority of compounds analyzed by gas chromatography, occur in the ultraviolet region, where flame background contributions are troublesome. Added to which, the diffusion flames used in gas chromatographic detectors lack sufficient stability and thermal energy to be useful atom reservoirs. Although, about 28 elements that includes phosphorus and sulfur, can be determined by their chemiluminescence emission in a hydrogen diffusion flame with a flame photometric detector (FPD) [337-339]. A number of chemiluminescence reaction detectors are also used in gas chromatography for the specific detection of sulfur- and nitrogen-containing compounds, nitrosamines and compounds capable of reducing nitrogen dioxide to nitric oxide [340]. For direct optical emission detection, microwave induced and inductively coupled plasmas provide more appropriate atom sources for organic compounds.

3.10.3.1 Flame Photometric Detector

The flame photometric detector (FPD) is an element-selective detector commonly used for the determination of sulfur- and phosphorous-containing compounds. The FPD uses a hydrogen diffusion flame to first decompose and then excite to a higher electronic state the fragments generated by the combustion of sulfur- and phosphorus-containing compounds in the effluent from a gas chromatograph. These excited state species subsequently return to the ground state, emitting characteristic band spectra. This emission is isolated by a filter and monitored by a photomultiplier detector. The FPD was originally described as a single flame detector [252,194,253,341] but is now available as a dual flame [342,343] and a pulsed flame version [344,345].

In the single flame detector design the carrier gas and air or oxygen are mixed, conveyed to the flame tip, and combusted in an atmosphere of hydrogen. With this burner and flow configuration interfering emissions from hydrocarbons occur mainly in the oxygen-rich flame regions close to the burner orifice, whereas sulfur and phosphorus emissions occur in the diffuse hydrogen-rich upper portions of the flame. To enhance the selectivity of the detector an opaque shield surrounds the base of the flame, minimizing the hydrocarbon emissions reaching the photomultiplier-viewing region. For any given burner design, the response of the FPD is critically dependent upon the ratio of hydrogen to air or oxygen flow rates, the type and flow rate of carrier gas and the temperature of the detector block [341,346,347]. Different optimum conditions are usually required for sulfur and phosphorus detection, for detectors with different burner designs, and perhaps also for different compound classes determined with the same detector. The sulfur response may decrease substantially at high carrier gas flow rates, more so with nitrogen than helium, but less response variation has been noted for phosphorus.

Problems with solvent flameout, hydrocarbon quenching and structure-response variations for different sulfur- and phosphorus-containing compounds with the single flame detector can be partially overcome using a dual-flame configuration, Figure 3.23

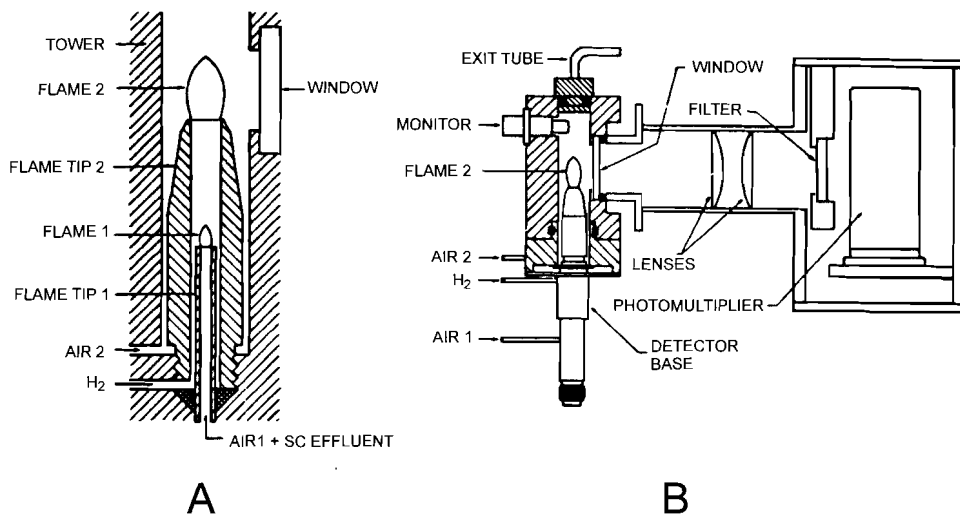


Figure 3.23. Cross-sectional view of the dual-flame burner (A) and schematic diagram showing the relationship between the burner and the photometric viewing components (B). (From ref. [342]; ©American Chemical Society).

[342,343]. The lower flame is hydrogen rich and functions as a matrix-normalization reactor in which all compounds are decomposed to a highly reduced state (e.g. H₂S, S₂, H₂O, CH₄, etc). The combustion products from the first flame are swept into a second longitudinally separated flame where the desired optical emission is generated under optimized flame conditions. The small detector volume, 170 μ l, and the high carrier and combustion gas flow rates in the passageways between the burners provide a very small effective detector dead volume, suitable for use with open tubular columns. Although sulfur detection limits are generally lower for the single flame detector, the dual flame detector provides a more reproducible response, less dependent on structure and the presence of coeluting hydrocarbons.

The pulsed-flame detector is based on a flame source and flame gas flow rates that cannot sustain a continuous flame. The combustion gases (H₂ and air) are mixed together in a small chamber and flow to a continuously heated wire igniter. The ignited flame then propagates back to the gas source and is self-terminated once all of the combustible gas mixture present in the combustion path is consumed. The continuous gas flow removes the combustion products and creates additional ignition in a periodic fashion (10^2 - 10^3 milliseconds). The pulsed flame emission is imaged onto a photomultiplier detector through a sapphire window, quartz light guide and optical filter. The pulsed flame emission provides enhanced detection sensitivity and selectivity by time resolution of the various flame luminescent species. The luminescence from hydrocarbon and flame combustion products (e.g. OH*, CH*, C₂* etc.) is limited to the time duration for flame front to pass across the photomultiplier viewing area. Because

of lower bond energies the sulfur- and phosphorous-containing species continue to emit in the cooler, yet reactive, post pulse flame conditions. Consequently, the heteroatom emission can be time resolved from the hydrocarbon and flame background emission with a gated detector. Other elements besides sulfur and phosphorous that show time-delayed emission include N, As, Se, Sn, Ge, Ga, Sb, Te, Br, Cu and In [338]. In addition thirteen other elements (Mn, Fe, Ru, Rh, Cr, Ni, Eu, V, W, B, Si, Al, Pb and Bi) exhibit flame chemiluminescence without delayed emission [337,338]. Thus, the FPD could potentially be used to determine a wider range of heteroatoms than is currently practiced.

The response mechanism of the FPD to sulfur- and phosphorous-containing compounds is known superficially, even if the finer details remain obscure. In the relatively low temperature (< 1000°C) and hydrogen-rich FPD flames, sulfur-containing compounds are decomposed and interconverted by a large set of bimolecular reactions to species, such as H₂S, HS, S, S₂, SO and SO₂ in relative proportions that depend on the instantaneous and fluctuating flame chemistry. In the presence of carbon radicals in the flame, various carbon-sulfur containing species might also be anticipated. Excited state S₂* species could result from several two or three body collision reactions, such as those shown below



where M is some third body. Only a small fraction of sulfur entering the flame is converted to S₂ species. The relaxation of S₂* results in broad band emission over the wavelength range from 320 to 460 nm with a maximum emission at 394 nm. In the case of phosphorus, phosphorus-containing compounds are first decomposed to PO molecules, which are then converted into HPO* by either of the reactions shown below



A linear dependence between detector response and the amount of sample entering the detector is expected for phosphorus. The response for sulfur is inherently non-linear and is described by $I(\text{S}_2) = A [\text{S}]^n$, where $I(\text{S}_2)$ is the detector response, A an experimental constant, [S] the mass flow rate of sulfur atoms, and n an exponential factor. The theoretical value for n is 2, but in practice, values between 1.6 and 2.2 are frequently observed for the single flame FPD. Non-optimized flame conditions, compound-dependent decomposition, hydrocarbon quenching, and competing flame reactions that lead to de-excitation all contribute to this deviation. Decoupling the

compound decomposition process from the excitation process in the dual flame and pulsed flame detectors results in a more truly quadratic response. If column performance parameters are determined with a FPD, it should be noted that equations derived for a linear detector response have to be modified [348]. For example, when $n = 2$, the peak width at one-quarter peak height is equivalent to the peak width at half height determined with a linear response detector.

Minimum detection limits and selectivity values for the FPD depends on the operating conditions, flame type and photomultiplier response characteristics. Typical detection limits are 5×10^{-13} to 1×10^{-14} g P/s (all detector types) and 5 to 50×10^{-12} g S/s (single flame), 1×10^{-11} g S/s (dual flame) and 2×10^{-13} g S/s (pulsed flame). The linear range for phosphorus usually exceeds 10^3 while the selectivity is more than 5×10^5 g C/g P. Sulfur selectivity varies from 10^4 - 10^6 g C/g S (single flame), 10^3 - 10^4 g C/g S (dual flame) and $> 10^7$ g C/g S (pulsed flame). The linear dynamic response range for sulfur based on an exponential factor of 2 is about 10^2 - 10^3 . The coelution of hydrocarbon compounds can quench the response of the FPD to sulfur-containing compounds, in particular [341,349-352]. Although the exact mechanism of quenching is unknown, it appears that both the change in the flame temperature as well as the flame chemistry that occur when large amounts of hydrocarbons are introduced into the flame play a role. A likely major contributing factor is nonradiative collisional deactivation of S_2^* by CO_2 , CH_4 , and other combustion products. The dual flame and pulsed flame FPD are less influenced by quenching than the single flame detector.

3.10.3.2 Chemiluminescence Detectors

Chemiluminescence is the term used to describe chemical reactions that result in the formation of vibrationally or electronically excited species that subsequently undergo photon emission. Those reactions important for gas chromatography occur in the gas phase between ozone and specific fragments produced by the thermal or catalytic decomposition of compounds eluting from the column [34,340,353-355]. Nitrosamines are detected after thermal cleavage and nitrogen-containing compounds after oxidation to nitric oxide, which reacts with ozone to form nitrogen dioxide in an excited state with photon emission in the near infrared around 1200 nm.



In the redox chemiluminescence detector (RCD) the column effluent is first mixed with a gas stream containing 100 to 300 ppm of nitrogen dioxide and then passed through a thermostatted catalyst chamber containing gold-coated glass beads or a metal oxide [340,353]. In the catalyst chamber any compound that reduces nitrogen dioxide to nitric oxide, produces a surrogate pulse of nitric oxide, which is subsequently detected by reaction with ozone. The selectivity of the reaction is controlled by the choice of catalyst, temperature and the catalyst support material. The response of the RCD covers

about 6 orders of magnitude and is suitable for the trace analysis of certain compounds not easily determined by other detectors due to either a poor response or poor selectivity.

Sulfur-containing compounds are decomposed by thermal oxidation to sulfur monoxide, which is stabilized in a reducing environment and subsequently reacted with ozone to form sulfur dioxide in an excited state with photon emission centered around 360 nm (range 280-460 nm).



A typical chemiluminescence detector consists of a series coupled thermal reaction and ozone reaction chambers. The selective detection of nitrosamines is based on their facile low temperature (275-300°C) catalytic pyrolysis to release nitric oxide. For nitrogen-containing compounds thermal decomposition in the presence of oxygen at about 1000°C is generally used for conversion to nitric oxide, although catalytic oxidation at lower temperatures is possible. For sulfur-containing compounds decomposition in a hydrogen diffusion flame or a ceramic furnace is used. The flame reactor employs a hydrogen rich flame where decomposition occurs in the hot reaction zone of the lower part of the flame and production of the chemiluminescent species in the reducing environment of the outer regions of the flame. A ceramic sampling probe mounted above the flame transports the combustion products from the flame to the ozone reaction chamber. The ozone reaction chamber and sampling probe are maintained at reduced pressure enabling about 90% of the flame combustion products to be transferred to the reaction chamber. In the furnace reaction chamber the column effluent is mixed with oxygen and flows into a thermostatted ceramic tube (750-1000°C) with a counter flow of hydrogen introduced at the opposite end. This creates a highly oxidative environment for decomposition in the lower portion of the furnace. The oxidant is largely depleted by the time the reaction mixture reaches the middle portion of the furnace trapping the decomposition products in a hot reducing gas stream. A modification of this process uses a concentric tube furnace in which oxidation occurs in the shorter inner tube at the lower part of the furnace and the subsequent reduction takes place in the outer ceramic tube at the upper part. In both cases the furnace is at a reduced pressure facilitating direct connection to the ozone reaction chamber. In all cases the hydrogen-to-oxygen (or air) ratio is an important operating parameter. There is some evidence that the catalytic activity of the ceramic surfaces contributes to the efficiency of the conversion process. The reactive sulfur-containing species produced by the flame and furnace reactors are believed to be the same. In addition to sulfur monoxide, it has been suggested that sulfur atoms produced in the conversion process combine with S₂ within the transfer line to the reaction chamber forming S₃, which reacts rapidly with ozone in the reaction chamber to form sulfur monoxide, initiating the chemiluminescence reaction [356,357].

The products of the conversion reaction flow into the ozone reaction chamber, which is maintained at a pressure of 10-30 mm Hg. Ozone is generated by a high

voltage discharge in air or oxygen and enters the reaction chamber by a separate connection. Reduced pressure operation has three advantages: it improves sensitivity by diminishing collisional deactivation of the excited state reaction products; it prevents condensation of water in the reaction chamber; and it reduces the effective detector dead volume maintaining compatibility with the use of open tubular columns. The chemiluminescence emission is isolated by an optical filter and detected by a photomultiplier. If one of the reactants in the chamber is maintained in large excess (e.g. ozone), the reaction becomes pseudo first order for the other reactant, providing a linear response mechanism for the detector.

The most important applications of the chemiluminescence detector are the determination of volatile nitrosamines and sulfur-containing compounds, often in complex matrices. Detection limits of about 0.5 ng, a linear response range up to 10^5 and the ability to discriminate against most amines and nitro compounds in higher concentration ($>10^3$) make this a very useful detector for the determination of carcinogenic nitrosamines in food, environmental extracts and industrial products. Unlike the flame photometric detector, the response of the chemiluminescence detector to sulfur-containing compounds is almost equal on a per gram of sulfur basis. Therefore, it is generally feasible to use a single calibration curve for the determination of all sulfur-containing compounds. The detection limit for the flame based detector is about 10^{-12} g S/s, with a linear range 10^4 - 10^5 , and selectivity $> 10^6$ g C/g S. For the flameless detector the detection limit is about 10^{-13} g S/s, with a linear range 10^4 - 10^5 , and selectivity $> 10^7$ g C/g S. The sulfur chemiluminescence detector is widely used in the petroleum and gas industries for process control and regulatory compliance, and in the beverage industry to characterize flavor compounds [34, 340,353,358,359]. In the nitrogen-selective mode the chemiluminescence detector has a detection limit of about 10^{-12} g N/s, a linear response range of 10^4 - 10^5 , and selectivity 10^7 g C/g N. It is more expensive and about an order of magnitude less sensitive than the thermionic ionization detector, which is more widely used for the selective determination of nitrogen-containing compounds. Unlike the thermionic ionization detector operated in the nitrogen mode, the nitrogen chemiluminescence detector is relatively unresponsive to phosphorous.

3.10.3.3 Atomic Emission Detector

Early studies employing atomic emission detection used simple interfaces to flames, carbon furnaces or plasmas as atom sources with standard or modified atomic spectroscopic instruments [360]. These systems were more or less restricted to the selective detection of metal-containing compounds. Direct current or inductively coupled argon plasmas effectively decompose organic compounds but provide poor excitation efficiency for the nonmetallic elements of primary interest for gas chromatography. Helium plasmas provide higher excitation energy and a simple background spectrum that allows all elements of interest to be detected. Atomic emission detection, however, was little used outside of a few research centers until Hewlett-Packard (now Agilent Technologies) introduced an automated, atmospheric pressure helium plasma, multi-element detector in about 1989 known as the AED [7,34,254,361-364]. This detector was responsible for

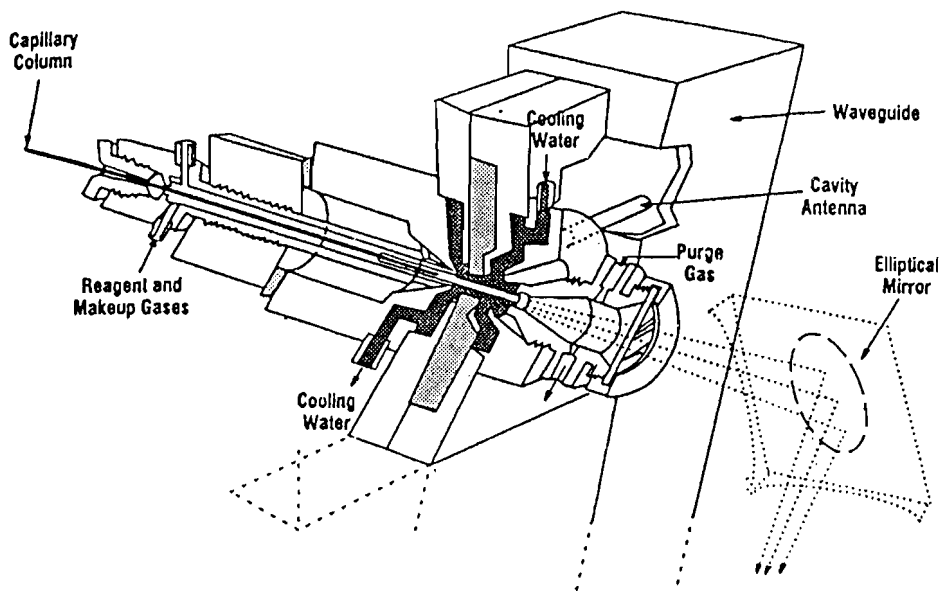


Figure 3.24. Cutaway view of the atomic emission detector cavity block. (From ref. [363]; ©Wiley-VCH).

generating general interest in atomic emission as a universal element-selective detector for gas chromatography [365-367]. The atomic emission detector underwent a significant hardware and software upgrade in about 1996, resulting in improved sensitivity and selectivity for several elements, such as nitrogen and sulfur [368,369].

In the basic AED setup, the separation column is fed from the gas chromatograph to the entrance of the microwave cavity through a heated transfer line. Coupling of the separation column to the heated cavity block (up to 400°C) is made through the gas flow system, which controls the flow of carrier gas, makeup gas and scavenger gases (O_2 , H_2 and CH_4) into the cavity. The plasma is produced in a thin-walled silica discharge tube within a microwave "reentrant" cavity, Figure 3.24. The discharge tube is water cooled to minimize erosion. Power is supplied by a magnetron, and coupled to the plasma through a waveguide. The exit of the cavity is closed with a fused silica window and purged with helium to prevent back diffusion of air into the cavity, and to allow flow reversal so that the solvent peak can be vented in front of the cavity. Solvent venting helps to minimize the build up of carbon in the cavity. The plasma is generated in an atmospheric pressure flow of helium made up of the column flow and additional makeup flow, as required. Depending on the elements being determined, low concentrations of various scavenger gases are also added. The cavity is attached to the outside wall of the spectrometer. An elliptical mirror collects emissions from 2 mm inside the end of the discharge tube. The emission sensor consists of a flat focal-plane spectrometer with a movable photodiode array containing 211 diodes. The spectral resolution of the AED is about 0.25 nm (full width at half maximum). The photodiode array moves along the

Table 3.5

Response characteristics of the atomic emission detector to different elements

Element (X)	Wavelength	Minimum detectable amount (pg/s)	Selectivity (g X/g C)	Linear range
C	193.1	2.6		2×10^4
H	486.1	2.2		6×10^3
Cl	479.5	39	2.5×10^4	2×10^4
Br	470.5	10	1.1×10^4	1×10^3
F	685.6	40	3.0×10^4	2×10^3
S	180.7	1	3.5×10^4	1×10^4
P	177.5	1	5.0×10^3	1×10^3
N	174.2	15	2.0×10^3	4×10^3
N	388	15	8.0×10^5	1×10^4
O	777.2	50	3.0×10^4	3×10^3
Sn	303.1	0.5	3.0×10^4	1×10^3
Se	196.1	4	5.0×10^4	1×10^3
Hg	253.7	0.1	3.0×10^6	1×10^3

focal plane, which is nearly linear from 160 to 800 nm. The array range is about 25 nm, which determines which element combinations can be measured simultaneously.

All functions of the AED system are keyboard controlled. At startup the wavelength is automatically calibrated using the atomic emission lines of helium as a reference and the photodiode array moved to a position in the focal plane where the element(s) of interest have emission lines. The correct scavenger gases (H_2 , O_2 , or CH_4) are automatically turned on and the required order shorter (optical filter) selected by the software. In order to analyze elements simultaneously, they must have emission lines which fall within the wavelength range spanned by the photodiode array and require the same scavenger gases. In general, up to four elements can be detected simultaneously and displayed as element specific chromatograms. Some common combinations of elements cannot be recorded simultaneously because of the relative position of their principal emission lines or incompatibility with the scavenger gas (e.g. C, S and N or C, H, Cl and Br can be detected simultaneously but O, P, and F could not be determined for the same conditions). Individual element detection limits range from 0.1 to 75 pg/s with a linear range of about 10^3 - 10^4 , Table 3.5.

The AED is a complex detector and its response depends on many parameters. Individual element conditions are optimized according to software "recipes" or developed by the operator and stored in memory [361-364]. The AED can be used to detect labeled compounds containing stable isotopes. Only the hydrogen isotopes atomic spectra can be resolved, but carbon and nitrogen isotopes can be distinguished by their molecular emission spectra following reaction in the plasma to produce CO and CN, respectively [370]. The addition of carbon compounds to the plasma (e.g. CH_4) increases the emission from molecular species, such as CN, and reduces background interference. For nitrogen the detection of CN is often preferable to detection at the atom emission line [369]. A large number of factors can affect the AED response at

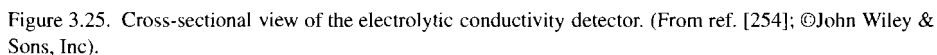
any particular wavelength including the concentration of scavenger gas, the flow rate of makeup gas, changes in the carrier gas flow rate, modification of the surface activity of the discharge tube, fluctuations in the power input, etc. [360,361,368,371-373]. Any factor that affects the temperature, composition and sample residence time in the plasma is a potential source of poor reproducibility. These problems are minimized as far as possible by a high level of detector automation.

At least in theory, all molecules should be completely decomposed into atoms and their derived ions at the plasma temperature. In which case the response for each element should be proportional to the number of atoms in the plasma and independent of compound structure. After response standardization with compounds of known elemental composition it should be possible to determine the empirical formula for each peak in the chromatogram [367,373-376]. In addition, it should be possible to use a single compound containing all elements of interest as an internal standard for calibration in quantitative analysis. The accuracy of such measurements is, however, limited by a lack of plasma stability, incomplete compound destruction, and deviations from linearity of the individual element responses. Accurate formula values for oxygen and nitrogen can be particularly difficult to determine due to entrainment of atmospheric gases into the plasma. Hydrogen, fluorine and phosphorus show significant discrepancies due to surface chemical interactions with the discharge tube. In the general sense it must be concluded that empirical formula calculations and the use of a single compound internal standard for quantification is not always possible with the desired accuracy. The reliability of empirical formulae calculations using the AED can be considerably improved by combining the information from element response ratios with molecular weight information obtained by mass spectrometry [367]. For the most accurate quantitative analysis a reference compound similar to the analyte in terms of elemental composition, molecular structure, physical state and concentration is required.

3.10.4 Electrochemical Detectors

There are two general problems as far as the application of electrochemical detection in gas chromatography is concerned. First of all, few electrochemical detectors are gas phase sensing devices, and therefore the separated sample components must be transferred into solution for detection. Secondly, the majority of organic compounds separated by gas chromatography are neither electrochemically active nor highly conducting. The electrolytic conductivity detector (ELCD) solves both of these problems by decomposing the gas phase sample into small inorganic molecules, which are detected by their conductivity in a support solvent [254,377]. The ELCD is used primarily as an element-selective detector for halogen-, sulfur- and nitrogen-containing compounds.

The column effluent is mixed with a reaction gas at the detector base and then passed through a small diameter nickel tube in a microfurnace at 850-1000°C. The nickel tube acts as catalyst for the decomposition reaction. With hydrogen as the reaction gas, halogen-containing compounds are converted to hydrogen halide (HCl, HBr),



The reaction products from the furnace are swept into a gas-liquid contactor where they are mixed with an appropriate solvent, Figure 3.25. For example, 1-propanol for hydrogen halides, water containing 1-10 % v/v *t*-butyl alcohol for ammonia, and

methanol containing 1-5 % v/v water for sulfur dioxide. The support solvent must have low background conductivity and promote extensive ionization of the reaction species while minimizing the ionization of interfering compounds. From the gas-liquid contactor the support solvent flows to the conductivity cell, where detection takes place, either after separation of the liquid from insoluble gases, or as a mixed phase, depending on the detector design. A square wave bipolar pulse is used for the conductivity measurement to minimize capacitive contributions to the detector signal [378,379]. The support solvent is usually circulated through a closed system containing beds of ion exchange resins to purify and condition the replenished solvent. Conditioning may involve pH modification to improve the sensitivity and selectivity for a particular element [254].

The practical problems most frequently encountered in operating the ELCD are loss of response, excessive noise, poor linearity and poor peak shape [254,380-382]. Poor linearity is usually caused by neutralization of the conducting species when the pH of the support solvent is incorrect and/or the scrubber is exhausted. Neutralization problems are recognized by a sharp dip in the baseline just prior to the peak and a negative dip after the peak, which gradually increases to the baseline. Peak tailing is often caused by a contaminated scrubber, contaminated transfer line from the furnace to the cell, deactivated catalyst, unswept dead volumes, or the presence of interfering, conducting reaction products that are not removed by the scrubber. The absolute flow rates of carrier, reactor, and makeup gases, and the flow rate stability of the support solvent influence the sensitivity of the detector. Since it is a concentration-sensitive detector, a greater response is anticipated at lower solvent flow rates; this increase will, however, be accompanied by an increase in noise, resulting from flow fluctuations. Excessive noise can result from impurities both in gases and support solvents, fluctuations in temperature, contamination of the reactor tube and transfer lines, and flow instabilities.

The ELCD is capable of high sensitivity and selectivity, although optimizing detector conditions and maintaining constant sensitivity at low sample levels can be troublesome. Detection limits on the order of 10^{-12} g N/s, 10^{-12} g S/s and 5×10^{-13} g Cl/s with a linear response range of 10^3 to 10^5 can be obtained. Selectivity varies with the heteroatom detected and the detector operating conditions. Values in the range 10^4 to 10^9 g C/g N, S or Cl are possible. Use of the ELCD has declined in recent years, probably because of the need for continuous maintenance and difficulties in operating the detector with narrow bore open tubular columns. It is used primarily for the determination of chlorine- and nitrogen-containing compounds in environmental applications and for the determination of sulfur-compounds in fuel products. For most cases other suitable detector options are available (except for certain regulatory methods that specify use of the ELCD) [195].

3.10.5 Series Coupled Detectors

There are many occasions when it would be useful to obtain more information about a sample than can be recorded with a single response mechanism detector without

having to rerun the sample. Detectors with orthogonal response characteristics allow the recording of element or structure specific ratios that provide greater certainty in compound identification or peak purity. Yet due to their mechanism and physical requirements, most popular detection modes cannot be made to operate in the same detection volume. The common exceptions are the AED and FPD detectors, which allow more than one element response to be recorded at the same time by using parallel optical channels. In other cases the series coupling of detectors is expected to provide higher sensitivity and reproducibility compared to using a postcolumn splitter connected to several detectors operated in parallel (section 3.9). The split ratio may change with flow and temperature variations imposed on the splitter by the separation conditions and sensitivity is compromised because each detector processes only a fraction of the separated sample. In addition, for series coupled detectors there are fewer problems in locating the same component peak in each detector chromatogram, since consecutively detected peaks are separated by a fixed time interval. If detectors are to be coupled in series then the first detector must be either (virtually) nondestructive (TCD, PID, ECD) or destroy the sample reproducibly with the liberation of at least one species detectable by the second detector. Also, ideally it should be possible to optimize the response of each detector independently. Typical arrangements for series coupled detectors include ECD-FID [383], ECD-RFID-FID [384], PID-ELCD [385], PID-ECD-FID [386], pulsed FPD-FID [387], FID-SCD [388,389], and TID-SCD [390]. Detectors are usually coupled with simple devices that allow for addition of makeup gas and detector support gases, as needed. A number of detector-coupling kits are commercially available.

The general use of series (or parallel) coupled detectors has been to record different channels of independent information as a method of qualitative identification [391,392]. The use of detector ratios for compound identification has declined with the development of low cost mass spectrometers that allow the use of more reliable identification methods. There are also practical problems related to long term stability of response ratios and their system dependence. Although rarely addressed in the literature, the chromatograms recorded with series coupled detectors often show greater peak shape deterioration than predicted from consideration of the additional coupled detector volume.

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4.1 INTRODUCTION

The term “liquid chromatography” encompasses a lexicon of separation techniques with a single common feature, that of a liquid mobile phase. Compared with gases liquids provide a greater variety of solvating capabilities with more scope for selectivity optimization, while gases have more favorable kinetic properties yielding higher efficiencies and shorter separation times. Consequently, separations in liquid chromatography are usually performed with a modest number of theoretical plates at an optimized selectivity achieved by appropriate selection of the separation mode, stationary phase structure, and mobile phase composition. Understanding the complex relationship between the last three terms is the key to understanding how separations occur in liquid chromatography.

A number of distinct separation modes are employed in liquid chromatography. A general classification can be made in terms of the distribution process, Figure 4.1. The main processes are interfacial adsorption, the basis of liquid-solid chromatography (LSC); restricted permeability of porous solids, the basis of size-exclusion chromatography (SEC); partition, the basis of liquid-liquid (LLC) and bonded-phase chromatography (BPC); electrostatic interactions with immobilized ionic groups, the basis of ion-exchange chromatography (IEC); and the structure-specific binding of biopolymers to immobilized molecular recognition sites, the basis of affinity chromatography (AC).

Inorganic oxides and porous polymers with various surface functional groups served the early needs of liquid chromatography. The development of chemically bonded phases in the early 1970s had a major impact on the general practice of liquid chromatography bringing about a change in emphasis among separation modes [1]. Bonded-phase chromatography (BPC) has almost completely replaced liquid-liquid chromatography (LLC), in which samples are separated by partition between two immiscible liquids, one of which is immobilized by coating onto a porous sorbent. A lack of genuinely immiscible solvent pairs, capable of forming stable separation systems, was the primary reason for the demise of liquid-liquid chromatography. Modern synthetic techniques allow the surface properties of inorganic oxides and porous polymers to be tailored for specific applications across all separation modes. An outstanding success was the development of hydrophobic surfaces for separations employing polar mobile phases (for most practical applications an aqueous solution) for reversed-phase chromatography (RPC). Reversed-phase chromatography is now the most popular separation technique in liquid chromatography, accounting for about two-thirds of all reported separations. Its popularity is due in large part to the growth of life-science applications as a proportion of all liquid chromatographic separations. In addition, it provides unmatched versatility for the separation of neutral, polar and ionic samples of a wide molecular weight range together with comparative ease of optimizing separation conditions. By exploiting secondary chemical equilibria in the mobile phase weak acids and bases can be separated by pH control using ion suppression chromatography (ISC). Permanently ionized compounds are separated by ion pair chromatography (IPC) using mobile phase additives with a complementary

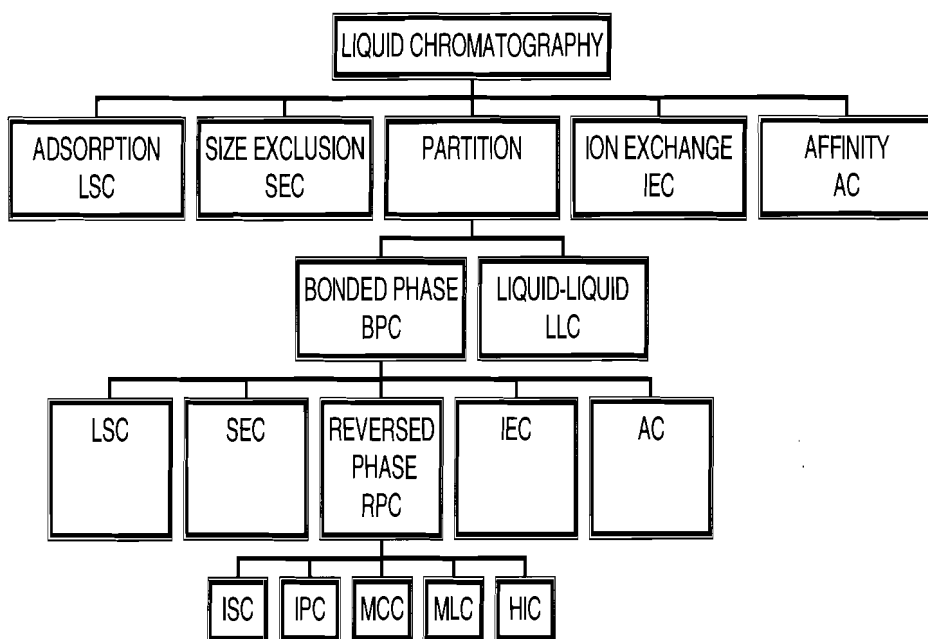


Figure 4.1. Family tree of liquid chromatographic separation modes. LSC = liquid-solid (or normal-phase) chromatography; SEC = size-exclusion chromatography; IEC = ion-exchange chromatography; AC = affinity chromatography; BPC = bonded-phase chromatography; LLC = liquid-liquid chromatography; RPC = reversed-phase chromatography; ISC = ion-suppression chromatography; IPC = ion-pair chromatography; MCC = metal-complexation chromatography; MLC = micellar-liquid chromatography; and HIC = hydrophobic-interaction chromatography.

charge to the analytes. Metal ions can be separated by formation of neutral complexes with suitable ligands added to the mobile phase by metal complexation chromatography (MCC). By adding a surfactant above its critical micelle concentration to the mobile phase, micelles can be used to modify the overall distribution constant of solutes between the mobile and stationary phases in micellar liquid chromatography (MLC). With a totally aqueous buffered mobile phase and a decreasing ionic strength gradient biopolymers can be separated with minimal disruption of conformational structure by hydrophobic interaction chromatography (HIC). Bonded-phase chemistry is also commonly used to prepare stationary phases with immobilized enantiomer-selective groups for the separation of racemates by chiral chromatography (section 10.4).

4.2 COLUMN PACKING MATERIALS

For what is now commonly referred to as conventional liquid chromatography separations were performed at low pressures using totally porous inorganic oxide or porous

polymer materials with an average particle size of 30-200 μm . These materials had good sample capacity but the large particle size and broad size distribution, together with the slow diffusion of sample molecules through the deep pores, resulted in poor efficiency and long separation times. The introduction of pellicular particles (porous layer beads) in the 1960s could be considered as the first modern column packing for high-pressure liquid chromatography. These materials had particle diameters of 30 to 55 μm and consisted of a glass bead core onto which was fused a 1-3 μm porous inorganic oxide layer. The rigid core permitted operation at high pressures and the thin porous stationary phase layer improved mass transfer characteristics. The porous layer beads were used without modification for liquid-solid chromatography, and in bonded-phase and ion-exchange chromatography after surface modification by reaction with organosilanes.

The importance of porous layer beads in modern liquid chromatography declined rapidly with the introduction of totally porous silica microparticles with narrow size ranges and average particle diameters less than 10 μm in the early 1970s. Indeed, when totally porous microparticles are employed, the most attractive feature of pellicular particles, namely their short diffusion pathways, becomes an intrinsic part of the support, and the use of an impervious core is unnecessary for particles with sufficient intrinsic strength. Compared with porous layer beads, totally porous microparticles provided faster separations and an increase of up to an order of magnitude in column efficiency and sample capacity. In the 1990s pellicular packings resurfaced in the form of nonporous microparticles of 0.5-5 μm diameter (section 4.2.6) and superficially porous particles of 3-6 μm diameter with a 0.1-1 μm porous shell [2], for use in the separation of biopolymers. Here, their principal advantage was improved mass transfer obtained by eliminating the slow diffusion of the biopolymers through the tortuous pore structure of conventional packings. The low surface areas and sample capacity of these materials, although unfavorable for the separation of small molecules, are advantageous for the fast separation of biopolymers.

Totally porous microparticles can be prepared from a number of substances [3-10]. By far the most important are the inorganic oxides (particularly silica), porous organic polymers, porous graphitic carbon, celluloses, hydroxyapatite, and various polysaccharides. Porous inorganic oxides, polymers and graphitic carbon dominate the separation of small molecules and inert macromolecules. For the separation of biopolymers a wider range of substrates with an optimized surface chemistry and particle morphology for the preservation of biological activity are used as well as nonporous particles.

4.2.1 Mesoporous Inorganic Oxides

Porous silica (or silica gel) is by far the most important adsorbent for liquid-solid chromatography and is also the substrate used to prepare most chemically bonded phases, giving it a preeminent position in modern column technology [3-15]. Silica particles are mechanically strong and easily prepared in a wide range of particle size ranges and pore diameters suitable for chromatography. As a substrate for the

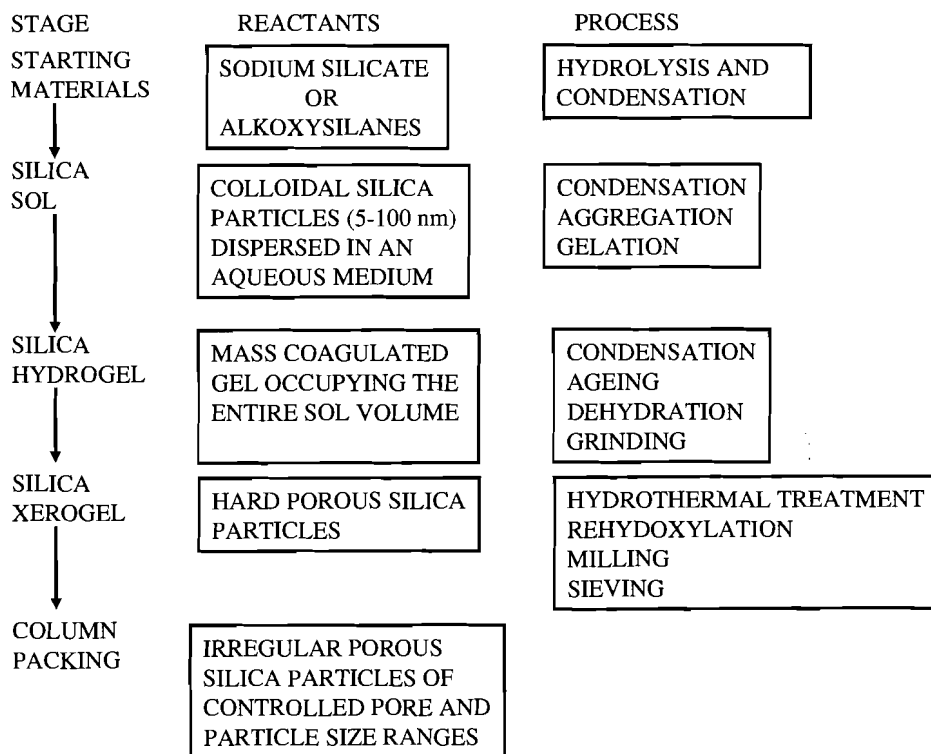


Figure 4.2. Schematic diagram of the different stages in the production of irregular porous silica particles by the sol-gel process for liquid chromatography.

synthesis of chemically bonded phases its reaction chemistry is well documented. For general applications in chromatography the main problems are its high solubility in alkaline solution ($\text{pH} > 8$), the limited hydrolytic stability of siloxane-bonded phases in acid solution ($\text{pH} < 2$) and strong interactions with basic compounds resulting in distorted peak shapes for both native silica and chemically bonded silica surfaces. To alleviate these problems other inorganic oxides (e.g. alumina, zirconia and titania) have been explored as well as the organic stationary phases described later in this section. Alumina, zirconia and titania are mechanically strong and more hydrolytically stable than silica with a complex, although complementary, surface chemistry to silica. For some applications they can be used in place of silica, but in general, they alleviate some problems only to replace them with a different set of problems (see section 4.2.1.2).

4.2.1.1 Silica

Granular or irregular porous silica microparticles are prepared by the conventional sol-gel process, as outlined in Figure 4.2 [4,6-10]. A silica sol is initially prepared by

the addition of sodium silicate or a tetraalkoxysilane to an aqueous acidic solution. Hydrolysis occurs spontaneously followed by condensation to poly(silicic acid) oligomers which, depending on the processing conditions, form discrete nonporous spheres of 5-100 nm in diameter with a characteristic size distribution. Aging of the silica sol results in coagulation of the colloidal silica particles to larger micrometer-sized aggregates accompanied by siloxane bond formation between particles and a strengthening of the gel framework. The continuation of this process (aging) gradually leads to mass coagulation and formation of an insoluble cake or hydrogel, which after crushing into suitably sized portions, is washed free of residual sodium salts, and finally dehydrated. A substantial shrinkage of the hydrogel pieces occurs upon dehydration, yielding a hard, porous xerogel that is subsequently milled and air sieved to the desired particle size range.

Spherical silica microparticles are more difficult to manufacture and can be prepared by several methods. One method (suspension gelation) prepares silica hydrogel beads by emulsification of a silica sol in an immiscible organic liquid [2,4-7,13,15]. The silica sol is dispersed into small droplets in a water immiscible organic solvent and the temperature, pH, and/or electrolyte concentration adjusted to promote solidification. Over time the liquid droplets become increasingly viscous and solidify as a coherent assembly of particles in bead form. The hydrogel beads are then dehydrated to porous, spherical, silica beads. Another route (suspension condensation) starts from a low molecular weight polymer, poly(ethoxysiloxane), prepared by partial hydrolysis of a tetraethoxysilane. The poly(ethoxysiloxane) oligomers are stirred in a water-methanol mixture to form a droplet suspension, followed by addition of catalyst (e.g. ammonia) to effect condensation and the formation of the corresponding silica hydrogel microbeads.

An alternative approach to the preparation of silica microparticles known as microencapsulation or polymer-induced colloid aggregation is based on the agglutination of a silica sol by coacervation [4,7,8,16,17]. Urea and formaldehyde are added to a well dispersed silica sol and the pH adjusted to effect the simultaneous condensation of the silica sol and organic monomers. Coacervated liquid droplets are formed containing a network of nanometer-sized silica particles encapsulated within a micrometer-sized network of organic polymer in the form of spherical beads that settle from the reaction medium and are subsequently harvested and dried. The polymeric binder is then burned out of the particles leaving porous silica microspheres. In this case, the mean particle diameter is controlled by the process variables and the pore diameter varied by using silica sols of different particle size ranges. As the pore diameter is increased the strength of the microparticles decreases. To compensate for this effect, and to maintain a minimum crush resistance, the larger pore size materials (50-400 nm) are processed at a higher temperature to increase the degree of sintering for strengthening purposes. Spherical silica microparticles and superficially porous particles can also be prepared by spray-drying techniques [4,6-8,18]. A silica sol with a defined concentration is dried in air at an elevated temperature by spraying from a nozzle into a centrifugal atomizer. During spray drying the liquid evaporates, leaving beads composed of compacted silica

particles. The spherical particles obtained in this way are then subjected to a hydrothermal treatment to adjust their porosity.

Different porous silicas are characterized by their particle morphology (shape, average particle size and pore diameter, surface area, pore volume and solubility) and surface structure (number and type of functional groups, the presence of impurities, and surface pH) [4,6-10,13,19-23]. For analytical separations of small molecules microparticles of 3 to 10 μm are the most widely used. These sizes provide a reasonable compromise between column performance, stability, operating pressure and separation time. Larger diameter particles, generally between 10 to 25 μm , are used in preparative-scale liquid chromatography. Still larger particles are used primarily for low-pressure column separations. Irrespective of the method of synthesis particles are produced in a size range characterized by a size distribution and an average particle size. To achieve a narrow size range the particles are usually classified after milling (irregular particles) or harvesting (spherical particles). For particles with an average size greater than 30 μm mechanical sieving is adequate. For smaller particles (3-30 μm) cross flow or counter flow air classification is required. In this case particles suspended in a gas stream are separated based on their settling velocity in a gravitational or centrifugal field. Several techniques (electrozone sensing, laser diffraction, sedimentation, microscopy, etc.) can be used to determine the particle size distribution. The resulting data are presented in one of three basic formats based on the number, area or volume (mass) distribution. Each distribution is a plot of the percentage of the particles in a narrow particle diameter window against the average particle diameter for that window. For the same material the cumulative statistical analysis of the data can lead to different mean particle diameters and size ranges, Figure 4.3. The volume and area distribution results in a larger average particle size than the number average and is generally more sensitive to the presence of outsize particles which may cause poor chromatographic performance. Since there is no agreed standard format for determining the mean particle size for chromatographic sorbents data from different sources are not necessarily comparable.

The pore structure of silica depends on the conditions of its synthesis and subsequent hydrothermal treatment. Treatment with water or water vapor at elevated temperatures (100-300°C) and possibly high pressures in an autoclave is used to optimize the pore size distribution of silica for particular applications. Under these conditions amorphous silica is transferred from the surface of large pores and deposited on the surface of the smaller pores. In this way the diameter of the larger pores is increased and the smaller pores filled in or blocked. The mean pore diameter, specific surface area and pore volume of porous silicas can be determined by well-established methods based on the sorption of gases or vapors, by mercury porosimetry, and less commonly, by electron microscopy [6,10,20,24-27]. The specific surface area can be separated into two components: the surface area within the pores and the external surface area of the particle. The pore surface area is several orders of magnitude larger than the external surface area and, in general, the larger the surface area of the packing material the smaller will be the mean pore diameter, Table 4.1. Ideally, the pore size distribution should be narrow and symmetrical about the mean value. Micropores are particularly

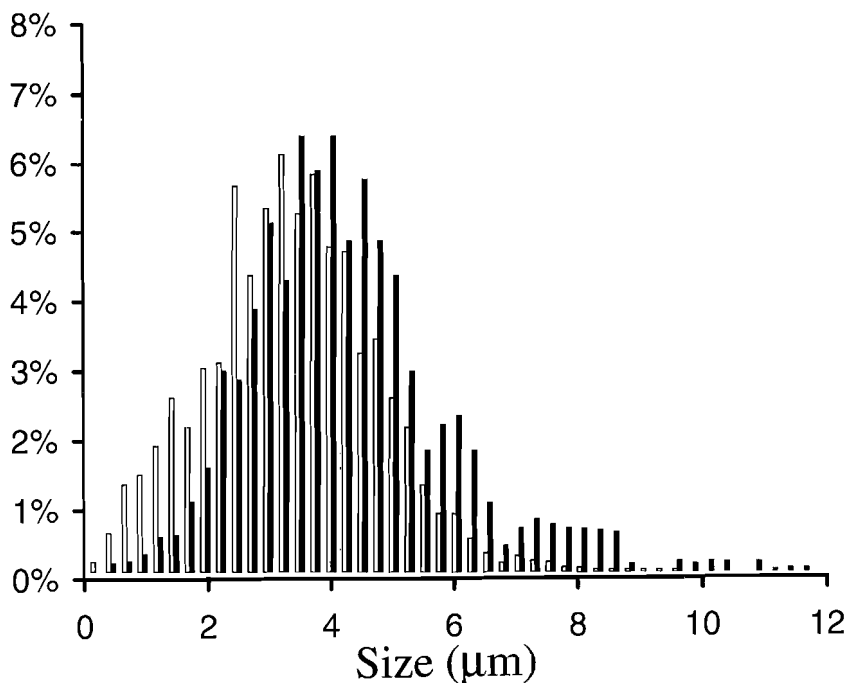


Figure 4.3. Particle size distribution for a 5 μm Spherisorb porous silica plotted as a number-average (open bars) and volume-average (filled bars) distribution. (Data from ref. [23]).

Table 4.1

Physical properties of Nucleosil porous silicas for liquid chromatography

Designation	Mean pore diameter (nm)	Pore volume (ml/g)	Specific surface area (m^2/g)
-50	5	0.8	500
-100	10	1.0	350
-120	12	0.7	200
-300	30	1.0	100
-500	50	0.9	35
-1000	100	0.75	25
-4000	400	0.70	10

undesirable, since they give rise to size-exclusion effects or irreversible adsorption due to the high surface energy existing within the narrow pores (micropores are considered to have a diameter < 2 nm, mesopores between 2-50 nm, and macropores > 50 nm; most types of chromatographic grade silicas are mesoporous but are often referred to as macroporous). To avoid chromatographic problems associated with narrow pores the mean pore diameter should be about ten times the critical dimension for the sample. As a result, porous silicas with mean pore diameters of 5-15 nm and surface areas of

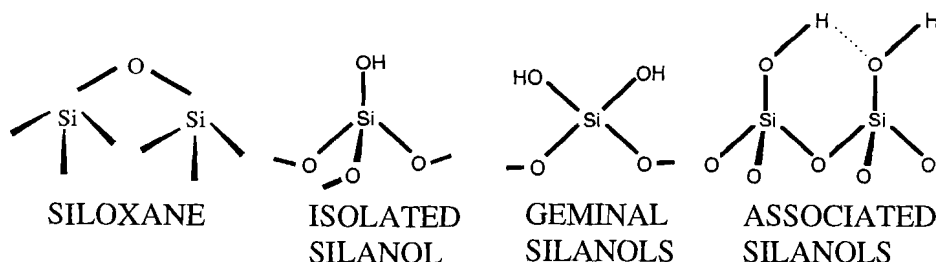


Figure 4.4. Characteristic functional groups on a silica surface.

150-600 m²/g are preferred for the separation of small molecules, while porous silicas with pore diameters greater than 30 nm are required for the separation of biopolymers.

It is important to note that of the above parameters only the specific pore volume, defined as the amount of liquid that fills the total volume of the pores per gram of adsorbent, has any physical significance. The specific surface area and the pore diameter are calculated by reference to an appropriate model and are only as accurate as the agreement between the model and the real situation. For porous silica particles a globular model is often, although not universally, used to estimate the mean pore diameter. This model assumes that the pore system can be described by a regular arrangement of densely packed spheres. The pore volume is formed by the crevices between the spheres and is controlled by the size and packing density of the spheres. The resulting pore space can be envisioned as a matrix of alternating wide cavities and narrow constrictions. The effective pore size distribution can also be determined by size-exclusion chromatography and may be more meaningful for chromatographic studies than values derived from liquid intrusion or gas sorption methods interpreted by phenomenological models (see section 4.5.4).

An understanding of the surface chemistry of silica is required to interpret its chromatographic properties. The silica surface consists of a network of single silanol groups (isolated silanols), silanediol groups (geminal silanols), hydrogen-bonded silanols (single or geminal), internal silanol groups, strained and stable siloxane bridges and rings, and physically adsorbed water hydrogen-bonded to all types of surface silanol groups, Figure 4.4. Isolated silanol groups include all hydroxyl groups located at a distance sufficiently far from neighboring hydroxyl groups to prevent hydrogen-bond formation. Associated silanols are silanol groups located on neighboring sites in which the hydroxyl to oxygen distance is sufficiently small that hydrogen bonding occurs. Internal silanol groups are located in the silica matrix and are inaccessible to external solutes. They are probably unimportant for chromatographic applications but complicate the interpretation of spectroscopic data used to infer chromatographic properties. The relative concentration and type of silanol groups on a dry silica surface can be determined by a combination of FTIR and ²⁹Si CP-MAS (cross polarization magic-angle spinning) NMR spectroscopy. FTIR spectroscopy can distinguish between isolated and associated silanol groups but not isolated and geminal silanol groups, which

are identified by NMR spectroscopy. The absolute concentration of all silanol groups can be determined by isotopic exchange reactions (preferred technique) or by reaction with a number of organometallic reagents. A fully hydroxylated silica surface contains about $8 \mu\text{mol}/\text{m}^2$ of silanol groups (4.8 silanols per nm^2). Many types of porous silica for liquid chromatography are not fully hydroxylated and have silanol group concentrations in the range $5.0\text{--}7.0 \mu\text{mol}/\text{m}^2$ depending on the method of preparation and subsequent treatment [10,13,20,28]. The surface concentration of silanol groups can be adjusted to the maximum level by rehydroxylation, for example, by boiling in water (very slow) or, by treatment with dilute aqueous hydrofluoric acid or tetra-*n*-butylammonium hydroxide [16,17,28-30].

From FTIR spectroscopy it is known that hydrogen-bond acceptor solutes are adsorbed preferentially on acidic, isolated silanol groups, whereas solutes with hydroxyl groups are adsorbed preferentially on associated silanol groups [13,31,32]. Kirkland suggested that porous silicas be divided into two categories for chromatographic applications, indicated as type A and B. Type A silicas have a low concentration of silanol groups while type B silicas are fully hydroxylated. The two categories being distinguished by the position of the silanol absorption band in the region of 3740 cm^{-1} [31]. FTIR evidence alone is probably inadequate to distinguish the two types [13] but the designation has proven useful and remains in use to describe silicas subjected to different treatments. Type B silicas have also become synonymous with the high purity silicas obtained, usually, from the hydrolysis of tetraalkoxysilanes. These porous silicas prepared with a highly hydroxylated surface contain low concentrations of impurities (e.g. $< 35 \text{ ppm}$ of metal ions) [30,33]. Type A silicas usually contain 0.1-0.3% (w/w) metal oxide impurities (chiefly, Na, Ca, Al, Mg, Ti, Ni, and Fe) [13,26,31]. It has been suggested that the indirect influence of matrix-incorporated metal impurities on adjacent silanol groups is responsible for their enhanced acidity. This would account for the small fraction of isolated silanol groups that react more strongly with basic solutes. Acid treatment can remove weakly attached surface contaminants but matrix-incorporated contaminants are more difficult to remove and probably persist after most treatments. Porous silicas with a low adsorptivity for basic solutes should possess a high concentration of associated silanol groups to minimize the number of isolated acidic silanols.

The surface composition of silica can be modified by thermal treatment [10,13,20,32]. Three temperature regions of importance include the region over which physically adsorbed water is removed (dehydration), a higher temperature region over which mainly associated silanols reversibly condense to siloxanes (dehydroxylation), and a third region, in which the temperature is high enough to induce lattice rearrangement and sintering occurs. Thermal gravimetric analysis and FTIR and ^1H CRAMPS (combined rotation and multiple pulse spectroscopy) NMR spectroscopy indicate that physically adsorbed water is removed completely by heating to about 200°C and is nearly complete by vacuum treatment at room temperature. Reversible dehydroxylation of silanol groups, resulting in an increased concentration of strained siloxane groups, commences at about 200°C , but is most efficient over the temperature range $400\text{--}600^\circ\text{C}$.

Thermal modification of silica below 400°C generally occurs with little change in the surface area and is readily reversible by boiling in water. FTIR measurements indicate that the associated silanol groups are removed preferentially, particularly above 400°C, and that isolated silanol groups can remain intact until much higher temperatures are reached. At temperatures above about 500°C, the siloxane groups formed from the dehydration of associated silanols, rearrange to a more stable configuration that is apparently less prone to silanol reformation. At temperatures in the range 600-1100°C sintering of the silica increases progressively accompanied by a significant decrease in the pore volume and a decrease in the concentration of isolated silanol groups (metal impurities strongly influence the absolute temperature at which sintering occurs). During sintering the silica becomes more fluid like and larger changes in physical and chemical properties are possible. A completely dehydrated silica surface is largely chemically and physically inactive and adsorbs moisture extremely slowly. It is not generally useful for chromatographic applications.

Theoretically, a fully hydroxylated silica surface should have a $pK_a = 7.1 \pm 0.5$. In practice, the apparent pH of porous silica materials used in liquid chromatography differ significantly from theoretical predictions and fall into the range 2 to 10 [12,16,33-35]. The apparent pH is determined by the observed pH of a 10 % (w/w) suspension of silica in neutral, salt free water. The wide range of apparent pH values for different sources of porous silica probably reflects differences in the manufacturing process, the presence of surface impurities, and variations in the relative concentration of different silanol groups. Although the apparent pH of silica has no real physical meaning, it has been shown to correlate reasonably well with the retention behavior of polar solutes using non-aqueous eluents. It was observed that “acidic silicas” were more efficient at eluting proton donor solutes while proton acceptor solutes could not be eluted in an acceptable time with reasonable peak shapes. The opposite is true for “basic silicas”.

The equilibrium concentration of amorphous silica in water at room temperature is about 100 ppm [36]. This value does not change much between pH 2-7. The solubility of silica increases exponentially above pH 8 due to the formation of the silicate anion. For mixed aqueous-organic eluents the solubility of silica depends on the aqueous content, type and concentration of buffer ions and temperature [13,37,38]. By using a high concentration of organic modifier, a saturator column before the injector, and ammonia as the source of hydroxyl ions, it was possible to use a silica column for a long time at a pH of 9.2 (given for the buffer solution). In another study, replacing ammonia by ethylenediamine permitted long term operation at pH 10.2 [39]. Under these conditions organic bases are retained by a combination of ion exchange and adsorption, often with high efficiency and good peak shapes. These conditions are not widely used in practice.

4.2.1.2 Alumina, titania and zirconia

Given the success of porous silica materials in liquid chromatography other inorganic oxides have been investigated in the hope of providing mechanically strong and more hydrolytically stable particles and bonded phases with a complementary surface chemistry [3,5,40-43]. Alumina (pH 2-12) and titania and zirconia (pH 1-14) are stable

to extreme pH conditions, which is there most attractive property when compared with silica. Porous alumina prepared by low temperature dehydration ($< 700^{\circ}\text{C}$) of alumina trihydrate has been used for many years in conventional liquid chromatography for separations and sample cleanup. These materials are a mixture of γ -alumina (more active form) and α -alumina, usually further modified by acid or base treatment to adjust the surface pH. Neutral alumina (pH 6.9-7.1) is the most widely used form; basic alumina (pH 10-10.5) is used to separate acid-labile compounds and in aqueous solution as a cation exchanger; and acid alumina (pH 3.5-4.5) is used to separate acidic substances and in aqueous solution as an anion exchanger. More refined materials are used in high-pressure liquid chromatography.

The methods used to synthesize irregular and spherical mesoporous alumina, zirconia and titania microparticles for high-pressure liquid chromatography are similar to those used to synthesize porous silica. While porous silica particles are amorphous these oxides are often prepared in several crystallographic forms, singularly or in mixtures, as well as amorphous materials. The degree of crystallinity and phase composition significantly influences the physicochemical and chromatographic properties of the oxides. Materials with a heterogeneous pore structure provide low chromatographic efficiency and the thermal history of a material is an important characteristic in judging its suitability for chromatography. With current manufacturing technology, alumina, zirconia and titania can be synthesized as microparticles with a mean diameter between 5-10 μm , a controlled pore diameter between 10-100 nm, and a specific surface area between 5-250 m^2/g . Spherical alumina microparticles with a unique morphology consisting of intersecting fused crystalline platelets with both macropores between the platelets ($\approx 0.1 \mu\text{m}$) and mesopores within the platelets (4-20 nm) has been described [44,45]. This material was designed to facilitate the use of columns at high linear velocities with a relatively low-pressure drop and has found more use for preparative-scale chromatography than for analytical separations. Porous zirconia [46-50] and titania [43,51,52] microparticles are prepared by suspension gelation in an oil-water emulsion or by microencapsulation using a urea-formaldehyde polymer. A colloid sol prepared by hydrolysis of a metal salt or tetraalkoxide provides the initial starting material. The zirconia and titania particles are sintered at high temperature to control the pore structure. In the case of zirconia, calcination in the presence of sodium chloride was shown to provide a suitable method for optimizing the pore structure, volume and surface area for chromatographic applications [48].

The surface chemistry of alumina, titania and zirconia are considerably more complex than silica [40,41,46,54-58]. Silica has only weak Bronsted acidity and cation exchange capacity associated with the presence of silanol groups. The surface chemistry of alumina, titania and zirconia is characterized by their acidity and basicity of a Bronsted and Lewis type and by their ion exchange properties. The metal oxide surface structure contains both oxygen and metal atoms with unsatisfied coordination valencies. The accumulation of negative charge on the oxygen atoms and positive charge on the metal atoms is the source of their ion exchange and Lewis ligand exchange properties. In normal-phase chromatography retention is determined largely by their Bronsted

basicity. Organic bases are usually separated with good peak shape (in contrast to silica) while acidic substances have long retention and often poor peak shapes. The Lewis acidity of the metal oxides increases in the order $\text{ZrO}_2 > \text{TiO}_2 > \text{Al}_2\text{O}_3$. Adsorbed inorganic polyanions (e.g. phosphate, carbonate, etc.) and fluoride modify the retention characteristics of zirconia by masking Lewis acid sites and reducing the retention of Lewis bases. The ion exchange capacity of the metal oxides increases in the order $\text{TiO}_2 > \text{ZrO}_2 > \text{Al}_2\text{O}_3$. The complexity of the retention mechanism and the influence of adsorbed species on the reproducibility of retention of metal oxides have resulted in only a limited number of practical, yet useful, applications.

4.2.2 Chemically Bonded Inorganic Oxides

Porous silica particles have good mechanical and kinetic properties but a limited range of selectivity. By modifying the silica surface with chemically bonded organic groups the selectivity of the particles can be tailored to a wider range of separation requirements. Silica-based, chemically bonded phases are used in all separation modes, Figure 1, and are the most important stationary phases for liquid chromatography. Their main limitations are poor stability at extreme mobile phase pH and the presence of accessible silanol groups after bonding that result in poor peak shapes and column performance for basic analytes. Modern column packing technology and choice of experimental conditions can minimize these problems.

A number of general reactions are used to prepare chemically bonded phases. The most important is the reaction between organosilane reagents and surface silanol groups with the formation of siloxane bonds [3,6,9,13,19,36,59-65]. Several research materials have been prepared by a two-step hydrosilanization/hydrosilation reaction [66]. In the first step the silica surface is reacted with the hydrolysis products of triethoxysilane to form a silica hydride intermediate which is subsequently reacted with a terminal olefin for attachment of the organic group through formation of a silicon to carbon bond. Older reaction strategies, such as reaction with an alcohol or isocyanate to form silicate esters (estersils) or chlorination of the silica surface with thionyl chloride followed by reaction of the silicon chloride with an organometallic reagent (e.g. Grignard, organolithium, etc.) are little used today. Silicate esters are too hydrolytically unstable to be of general use, and although bonded phases with a silicon-carbon bond are more stable than the silicate esters, the multi-step synthesis is demanding and requires scrupulously dry reaction conditions. In addition, reaction residues were often difficult to wash out of the final product. The replacement of a silicon atom in a siloxane bond by a heteroatom produces M-O-Si bonds (M = Al, Ti and Zr), which are less stable than the siloxane bond. The bonded phases formed by silanization of alumina, titania and zirconia are therefore not as useful as those prepared from porous silica [42,46,67,68]. More stable materials have been prepared from triethoxysilanes in the solution polymerization reaction and by the silanization/hydrosilation reaction. In both cases the metal oxide surface is covered by a siloxane polymer containing unreacted silanol groups as well as other active sites associated with the Lewis and Bronsted acid/base properties of the

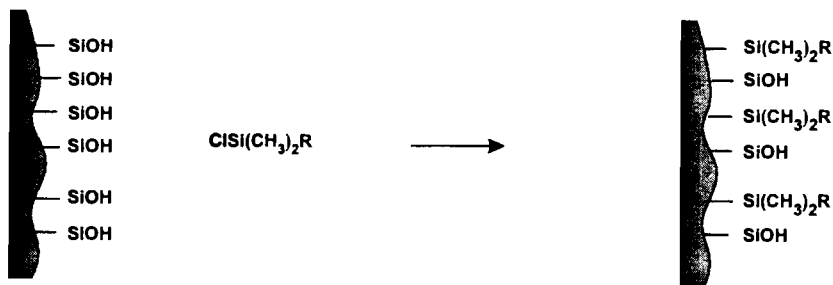
metal oxides. Because of these problems, polymer-coated metal oxides (section 4.2.3) seem to be a better practical alternative.

Organic-inorganic hybrid particles prepared by the sol-gel process were introduced recently to prepare silica-based materials with an extended pH and temperature operating range [65,69]. Only sketchy details of their synthesis are available. In general, a mixture of two organosilanes is used: one that forms silica units and another that forms methylsiloxane units. The resulting porous particles contain methylsiloxane groups throughout their internal and external structure. The presence of surface silanol groups allows a variety of organosiloxane-bonded groups to be introduced. These materials exhibit good chromatographic properties for a wide range of analytes at a mobile phase pH less than 12.

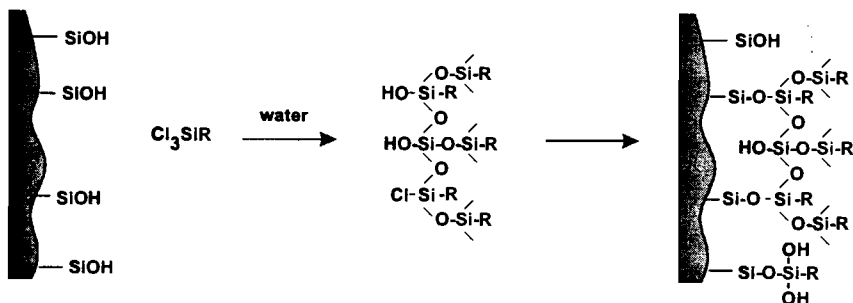
4.2.2.1 Organosiloxane-bonded phases

The majority of commercially available chemically bonded phases are based on a porous silica substrate modified by reaction with an organosilane reagent under carefully controlled conditions. The surface concentration of silanol groups depends on the method of synthesis and thermal history of the substrate. A fully hydroxylated silica substrate is preferred for the synthesis since it yields a final product with a higher bonding density, improved hydrolytic stability, increased mechanical strength, and a markedly lower adsorptivity for basic compounds [13,16,28,31,60,70-72]. Unless indicated otherwise, the silica is heated under vacuum for a few hours at less than 200°C to remove physically adsorbed water without dehydrating the surface prior to reaction. The bonding reaction is performed by refluxing the silica in an inert solvent with various reactive organosilanes. Monofunctional organosilanes under anhydrous conditions, usually in the presence of a catalyst, result in the formation of a monomeric bonded phase, Figure 4.5 [73]. Using a difunctional or trifunctional organosilane under similar conditions will also produce a monomeric phase with each ligand bonded to the surface by one or two bonds to the reagent-silicon atom [74]. This material will always contain some unreacted leaving groups, which must be endcapped during the workup. The addition of a controlled amount of water to a slurry containing silica and a difunctional or (usually) trifunctional organosilane heated to reflux results in the formation of a polymeric bonded phase [19,73,75,76]. Polymerization occurs in solution, with subsequent deposition on and reaction with the silica surface, as well as possibly further reaction within the immobilized polymer network, Figure 4.5. Solution polymerization typically results in a higher concentration of bonded phase than observed for the monomeric phases (i.e. 3-6 $\mu\text{mol}/\text{m}^2$ compared with 2-3.5 $\mu\text{mol}/\text{m}^2$), since siloxane bonds are formed within the polymer network projecting away from the surface (vertical polymerization) as well as with the surface. These phases also contain a large number of silanol groups, many of which arise from the hydrolysis of leaving groups that are not consumed in the polymerization process. A different type of polymeric bonded phase can be prepared by surface or horizontal polymerization of (usually) a mixture of long and short chain trichloroalkylsilanes at room temperature with wet silica [76-78]. The key parameter is the amount of water adsorbed on

Monomeric Synthesis



Solution Polymerization



Surface Polymerization

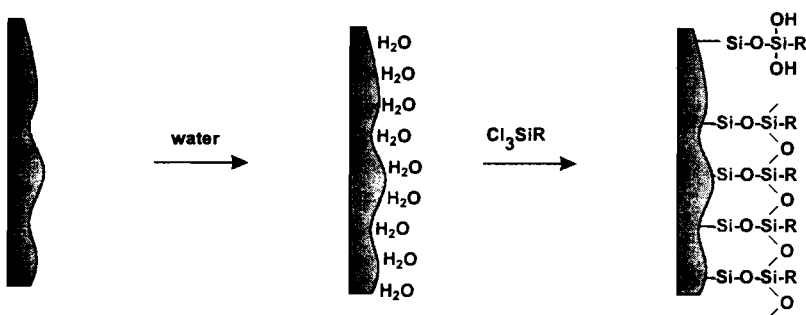


Figure 4.5. General outline for the synthesis of siloxane-bonded phases on a porous silica substrate. (From ref [73]. ©American Chemical Society).

the silica. Maximum surface coverage requires a stoichiometric amount of water to hydrolyze the organosilanes. The amount of water is usually controlled by equilibration of the silica with humidified nitrogen. The high surface concentration of immobilized polymer ($4.5\text{--}10\ \mu\text{mol}/\text{m}^2$) is unrelated to the surface silanol concentration since

there is little attachment to the surface, at least for the densely bonded phases. The high coverage results from the close-packed geometry of the horizontally assembled siloxane monolayer. A large number of defects exist in the monolayer created by incomplete crosslinking at sites where chlorosilane groups were unable to condense with neighboring silanol groups.

After the bonding reaction both monomeric and polymeric phases are washed with a series of organic and aqueous solutions to remove adsorbed reagent and to hydrolyze the remaining reactive functional groups that are part of the bonded phase skeleton. Since many undesirable chromatographic properties of bonded phases are associated with the presence of accessible silanol groups, their concentration is often minimized by reaction with an endcapping reagent following the initial preparation of the bonded phase [61,70,79-81]. Access to many of the silanol groups is sterically hindered and to maximize the extent of reaction the reagent should be small and is commonly a trimethylsilyl reagent such as trimethylchlorosilane or hexamethyldisilazane. Manufacturers rarely disclose the method used for endcapping. Some materials are described as double or triple endcapped without explanation. One method of double endcapping uses trimethylsilyl and dimethylsilyl reagents, but it is unclear whether this is as a mixture or in a sequential reaction [81]. Bonded phase materials for use with predominantly aqueous solutions are said to be endcapped with hydrophilic reagents but again without further details of structure or method [82]. In some separations the presence of silanol groups contributes to the success of the separation, and in these cases endcapping is undesirable. For this reason popular column packings are available in endcapped and non-endcapped versions.

Comparing the synthetic procedures discussed above, monomeric phases are straightforward to prepare and the reaction conditions should be more reproducible. The resulting monolayer coverage exhibits excellent mass transfer properties that produce high column efficiency for most solutes. Polymeric phases are more stable towards hydrolysis, but more difficult to prepare reproducibly and may also exhibit lower column efficiency. They are not as widely used as monomeric phases. A unique feature of polymeric phases is their shape selectivity. Compounds constrained to rigid conformations, such as polycyclic aromatic compounds, carotenoids, polychlorinated biphenyls and steroids are better separated by polymeric phases, roughly in order of their length-to-breadth ratios and planarity [73,75,76]. Conformationally flexible compounds generally exhibit similar retention behavior on monomeric and polymeric phases and similar methylene group selectivity.

A number of factors influence the success of the bonding reactions shown in Figure 4.5 [61,72,83]. The rate and extent of reaction depends on the reactivity of the organosilane, choice of solvent and catalyst, time, temperature and the ratio of reagents to substrate. Reactive organosilanes with Cl, OH, OR, N(CH₃)₂, trifluoroacetate, and enolate leaving groups have been widely used [61,70,83-86]. The dimethylamine, trifluoroacetate and enolates are the most reactive leaving groups but economy, availability and familiarity result in the chlorosilanes and alkoxysilanes being the most important, particularly for manufactured bonded-phase materials. Initial reactions may

be fast but as the surface coverage approaches a maximum value the rate of reaction declines. Reaction times, therefore, tend to be long, 12-72 hours, reaction temperatures moderately high (e.g. around 100°C), and in the case of chlorosilanes, an acid acceptor catalyst (e.g. pyridine or lutidine) is used. Some reagents, such as the alkylsilyl enolates and alkylsilyldimethylamines do not require additional catalyst, or even solvent, to carry out the reaction [81,84]. For the other reagents the most common solvents employed are toluene and xylene, although solvents, such as carbon tetrachloride, trichloroethane and dimethylformamide have been stated to be superior [83,85]. Since the bonding reactions are carried out by refluxing in an inert atmosphere, suitable solvents should be inert as well as a good solvent for the organosilanes, and be able to attain the desired reaction temperature at reflux. Except for 3-cyanopropylsiloxane bonded phases, the high reactivity of chlorosilanes towards certain polar functional groups (e.g. NH_2 , OH , CO_2H , etc.) precludes their use for the preparation of polar bonded phases. These are generally prepared from the less reactive alkoxysilanes [70,87]. Alkoxysilanes with ligands containing acidic or basic functional groups are autocatalytic. The bonded phases are usually prepared by refluxing the alkoxysilane in an inert solvent at a temperature high enough to distill off the alcohol formed by the condensation reaction with the surface silanol groups. Bonding of neutral, polar ligands generally requires the addition of a catalyst, such as toluene-4-sulfonic acid or triethylamine in the presence of sufficient water to generate monolayer coverage of the silica. The presence of water speeds up the hydrolysis of the alkoxy groups of the adsorbed organosilane, which tends to react with surface silanol groups rather than polymerize in solution. It seems to be a general problem in the preparation of polar bonded phases, that surface silanol groups are blocked by physically adsorbed organosilanes, giving rise to a lower bonded phase density after work-up than the maximum theoretically predicted. Repeating the reaction a second time increases the bonded phase density or endcapping can be used to mask accessible silanol groups. Diol bonded phases are prepared by refluxing glycidopropyltrimethoxysilane and silica in an aqueous suspension buffered to acidic or basic pH to promote bonding and simultaneous hydrolysis of the oxirane ring to a diol group [88,89]. Some bonding to silica via the terminal hydroxyl group may occur resulting in a mixed product. Bonded phases with an embedded amide [90,91], carbamate [91-93], or urea [94] group were developed to improve the chromatographic properties of basic analytes in reversed-phase chromatography. The embedded group acts by shielding the analytes from accessible surface silanol groups through internal hydrogen bonding between the embedded group and surface silanol groups. The embedded polar groups also promote the selective solvation of the silica surface by water, which results in improved shielding of the silanol groups and different selectivity to alkylsiloxane-bonded phases. Ion-exchange packings can be prepared from appropriately substituted alkoxysilanes, or by subsequent reaction or coupling of ligands containing ionic groups to a prebonded phase containing reactive functional groups [90]. Some typical examples of siloxane-bonded phases prepared from reactive organosilanes are summarized in Table 4.2 [3,19,61-63,70].

Table 4.2
Common substituents for siloxane-bonded phases

Type	Substituent	Application
Alkyl	-CH ₃	Reversed phase
	-C ₄ H ₉	
	-C ₈ H ₁₇	
	-C ₁₈ H ₃₇	
	-C ₃₀ H ₆₁	
Fluoroalkyl	-(CH ₂) ₂ (CF ₂) ₅ CF ₃ -(CH ₂) ₂ C(CF ₃) ₂ C ₃ F ₇	Reversed phase
Phenyl	-C ₆ H ₅	Reversed phase
	-C ₆ F ₅	
Cyano	-(CH ₂) ₃ CN	Normal and reversed phase
Amino	-(CH ₂) ₃ NH ₂	Normal and reversed phase.
		Weak anion exchanger
Diol	-(CH ₂) ₃ OCH ₂ CH(OH)CH ₂ (OH)	Normal phase and size exclusion
Amide	-(CH ₂) ₃ NHCOC ₁₃ H ₂₇	Reversed phase
Carbamate	-(CH ₂) ₃ OCONHC ₈ H ₁₇	Reversed phase
Sulfonic acid	-(CH ₂) ₃ SO ₃ H	Strong cation exchanger
	-C ₆ H ₄ SO ₃ H	
	-(CH ₂) ₃ C ₆ H ₄ SO ₃ H	
Carboxylic acid	-(CH ₂) ₃ OCH ₂ COOH	Weak cation exchanger
	-(CH ₂) ₃ COOH	
	-(CH ₂) ₃ C ₆ H ₄ CH ₂ COOH	
	-(CH ₂) ₃ N(CH ₃) ₂	
Dimethylamine	-(CH ₂) ₃ N(CH ₃) ₂	Weak anion exchanger
Quaternary amine	-(CH ₂) ₃ N ⁺ (CH ₃) ₃	Strong anion exchanger

It is generally recommended that silica-based, siloxane-bonded phases are restricted to use in the pH range 2 – 7.5. The main cause of column failure at low pH is the loss of bonded phase by hydrolysis of the siloxane bond attaching the organosilane to the silica surface. At intermediate and high pH column failure is due primarily to dissolution of the silica substrate. Modified organosilane reagents with steric protecting groups [86,95] and bidentate reagents [71,96] were introduced for the synthesis of siloxane-bonded phases with improved hydrolytic stability at extreme pH for use in reversed-phase liquid chromatography, Figure 4.6. Replacing the methyl groups in the alkyl dimethylsilane reagent with bulky isopropyl or isobutyl groups provides better steric protection of the siloxane bond to hydrolysis at low pH but is less successful at protecting the silica substrate at high pH. The bonding density achieved is lower (about 2 $\mu\text{mol}/\text{m}^2$) compared with conventional reagents on account of the larger size of the steric protecting groups at the silica surface. The superior stability of the bidentate bonded phases at low pH is a result of the high bonding density and the formation of two siloxane bonds with the silica surface. The bridging group, indicated by Q in Figure 4.6, is either -O- or -(CH₂)_n-, with -(CH₂)₃- being about optimum when R is an additional octadecyl group. Lower steric hindrance for reaction at the silica surface results in a higher bonding density and similar stability at low pH to the sterically protected bonded phases. For continuous operation at low pH siloxane-bonded phases based

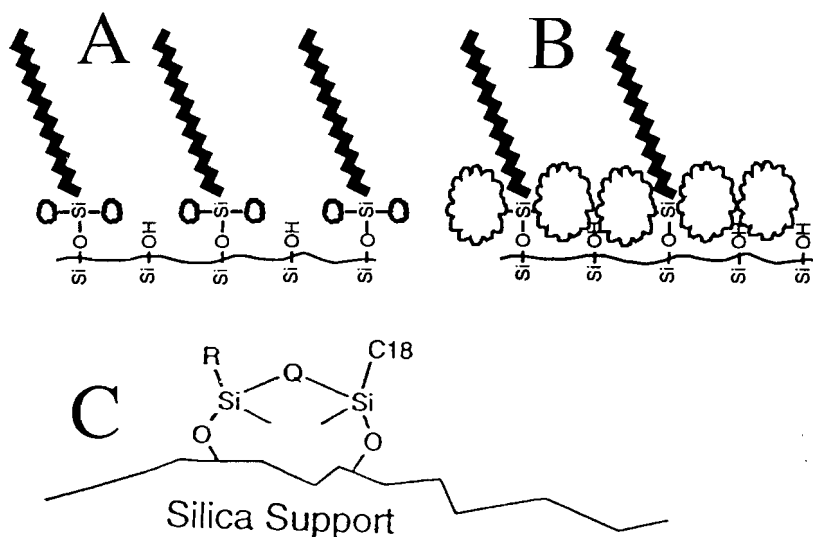


Figure 4.6. General structure of dimethylalkylsiloxane-bonded (A), sterically protected alkylsiloxane-bonded (B) and a bidentate alkylsiloxane-bonded (C) phases. For the bidentate phase $Q = -O-$ or $-(CH_2)_n-$ and R is an alkyl group.

on a fully hydroxylated pure silica (type B) synthesized with sterically protected or bidentate organosilanes are recommended [13]. For continuous operation at high pH (9–12) siloxane-bonded phases based on fully hydroxylated and low porosity (low surface area) silica made by aggregating silica sols are recommended [81]. These type A silicas are more stable at high pH than the high purity type B silicas. The bonded phase should be either bidentate or a densely bonded and fully endcapped alkyldimethylsiloxane-bonded phase. For both low and high pH operation bonded phases with a long chain alkyl group are more stable than bonded phases prepared with a short chain alkyl group. Mobile phases with a high proportion of organic solvent; the use of acetonitrile rather than methanol; the use of boric acid or organic buffers (glycine, citrate, pyrrolidine) in low concentration (≤ 50 mM); and low temperatures ($\leq 40^\circ\text{C}$), all contribute to column longevity at extreme pH [13,81,97,98]. Mobile phases containing phosphate or carbonate buffers are particularly aggressive in the dissolution of silica at $\text{pH} \geq 7$ and should be avoided. To extend the life of the bonded phase at high pH a precolumn containing silica placed prior to the injection valve can be used to condition the mobile phase for isocratic separations. Careful choice of bonded phase materials and operating conditions allows rugged reversed-phase chromatographic separations over the pH range 1–12 with acceptable column life times.

Mixed mode or zwitterionic siloxane-bonded phases have been prepared by co-bonding hydrophobic ligands, such as octyl or octadecyl, with cationic, anionic or polar ligands, in a single stationary phase [99]. An alternative approach is to first bond a ligand with a reactive terminal group, such as a 3-aminopropyl group, that

Table 4.3
Influence of silica morphology on the properties of octadecyldimethylsiloxane-bonded phases

Parameter	Phase	Value		
Mean pore diameter (nm)	Silica	10.5	18.8	25.0
	Bonded phase	8.5	16.2	21.6
Pore volume (ml/g)	Silica	1.13	1.30	1.36
	Bonded phase	0.53	0.80	0.39
Specific surface area (m ² /g)	Silica	423	261	204
	Bonded phase	176	147	136
Percent carbon	Bonded phase	19.2	15.47	13.2
Bonding density (μmol/m ²)	Bonded phase	2.52	3.09	3.25

can be used to introduce the desired chemical functionalities in a second reaction. For example, siloxane-bonded aminopropyl groups can be partially acylated with a reagent containing an ester function. Selective hydrolysis of the ester group to form a carboxyl group yields a phase containing both surface bonded amine and carboxylic acid groups. Various mixed mode bonded phases are commercially available, but are not widely used for separations. They have found greater acceptance for the isolation of drugs from biological fluids using solid-phase extraction.

There are three main factors that determine the accessibility of surface silanol groups for reaction with organosilane reagents. A steric effect, due to the fact that the cross-sectional areas of most bonded ligands is greater than the average spacing between surface silanol groups, differences in the reactivity and possible clustering of silanol groups, and the silica morphology, particularly its porosity. From spectroscopic evidence, it has been shown that the geminal and isolated silanol groups are the most reactive towards chlorosilanes [28,32]. Typical bonded phase coverage for monomeric phases corresponds to about 3.0-3.9 μmol/m² for octadecyldimethylsilyl ligands rising to a maximum of about 4.4 μmol/m² for trimethylsilyl ligands. Thus even at maximum surface bonding densities between 30 and 50 percent of the surface silanol groups will remain unreacted. From isotope exchange studies, it has been shown that access to all unreacted surface silanols is sterically hindered to different extents by the dense graft [100]. The bonding density also depends on the pore structure of the silica [13,61,75]. On passing from a flat to a concave surface the bonding density no longer depends solely on the size of the anchor group, but is determined by steric hindrance that occurs when the upper parts of the bonded ligands come into contact. This effect is of greater importance the longer the alkyl chain anchored to the pore surface. The extended conformation of the octadecylsilyl chain is about 2.45 nm long (for methylsilyl 0.3 nm, butylsilyl 0.7 nm, and octylsilyl 1.2 nm) and for pore diameters less than about 12 nm, assuming a cylindrical pore shape, the pore curvature will decrease the bonding density of the octadecyldimethylsiloxane ligands. In addition, initial reaction of the reagent at the edges of the cylindrical pores will limit diffusion of further reagent into the pores.

The initial physical properties of the silica substrate are also changed by the bonding reaction, Table 4.3. A significant reduction in the mean pore diameter, pore volume and specific surface area occurs after reaction with an organosilane[101].

There is a greater retention of the surface area and residual pore volume upon bonding as the pore diameter of the silica substrate increases. For similar bonding density the chromatographic retention decreases with increasing pore diameter as a consequence of the lower surface area, and therefore decreased quantity of bonded phase in the column. Selectivity differences, however, are expected to be minimal. More significant selectivity differences are likely for polymeric bonded phases if size exclusion effects limit the extent of surface modification during synthesis of the bonded phase [73,75,76]. Under the reaction conditions a mixture of organosilane monomer and polymer exist in solution. Both species can bond to the silica substrate. However, because the organosilane polymer is larger than the monomer, size differentiation within the pore network is possible. For small-pore substrates, a phase with a higher monomeric character results with lower shape selectivity. For large-pore substrates greater polymeric character is possible because more of the pore volume of the substrate is accessible to the reactive polymer.

A number of chromatographic (section 4.5.3) and physical or chemical methods are used to characterize the structural properties of siloxane-bonded phases [6,13,60,62,71,100,102]. The percent carbon loading for bonded phases is easily obtained by standard combustion analysis and is frequently quoted in the manufacturers' literature [103]. The percent carbon loading increases with the size of the bound ligand for the same surface coverage, but in the absence of specific knowledge of the surface area of the silica substrate, it is not a useful parameter for comparison between phases. A more useful parameter is the surface coverage of the bonded phase, α_{BP} , which for non-encapped phases can be calculated using Eq. (4.1)

$$\alpha_{BP} (\mu\text{mol}/\text{m}^2) = 10^6 P_c / [1200 n_c - P_c (M - n_x)] S_{BET} \quad (4.1)$$

where P_c is the percent carbon loading of the bonded phase, n_c the number of carbon atoms in the bonded ligand, n_x the number of reactive groups in the silane, M the molecular weight of the bonded ligand, and S_{BET} the surface area of the silica substrate [63,75]. For octadecylsiloxane-bonded phases prepared from trichlorosilanes, $\text{C}_{18}\text{H}_{37}\text{Si}(\text{OH})_2\text{O}-$ ($M = 331$) is taken as the representative unit for the phase structure. The chlorine atoms not consumed by bonding to silica are converted to silanol groups during the synthesis and work-up. The bonded phase coverage can be used to indicate the completeness of the bonding reaction for silicas with different pore structures and to estimate the concentration of unreacted silanol groups for bonded phases prepared from monofunctional organosilanes. The concentration of unreacted silanol groups can also be determined by deuterium exchange or by reaction with an organometallic reagent such as methylolithium [84,83,100,103-105]. The ligand type, method of bonding and reagents used for endcapping can be determined by cleavage of the siloxane bonds by alkali fusion in triglyme suspension [61] or by treatment with methanolic aqueous hydrogen fluoride [81,106]. Treatment with hydrofluoric acid produces stable, volatile organofluorosilanes, which can be identified by gas chromatography. Conventional and CP-MAS NMR of ^1H , ^{13}C , and ^{29}Si is now routinely used to identify the type of surface

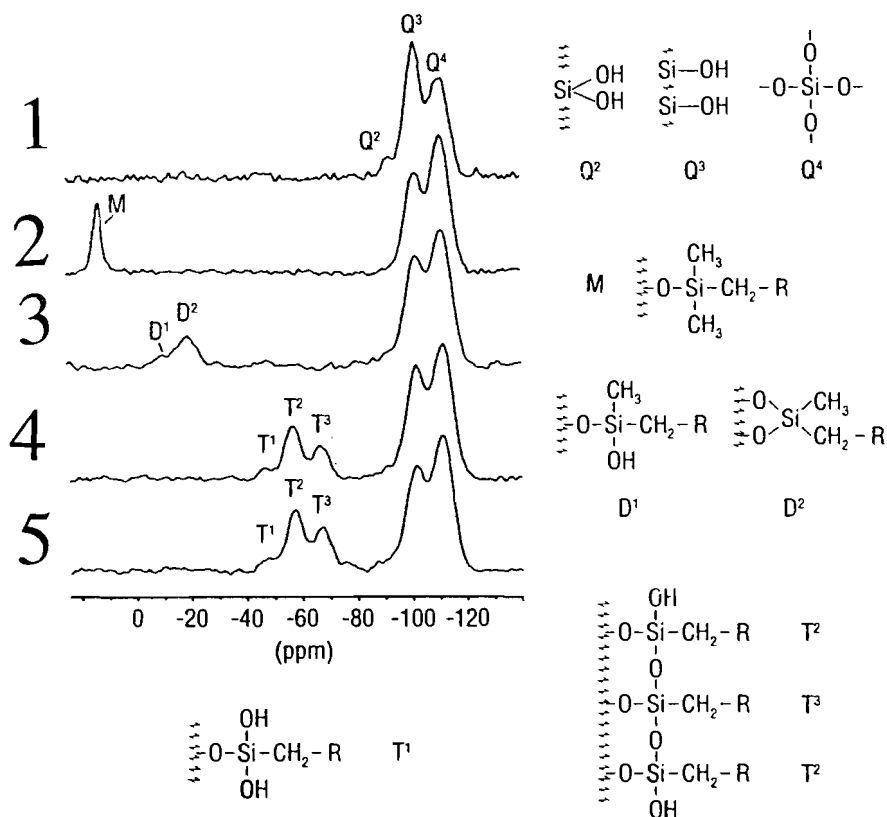


Figure 4.7. ^{29}Si CP-MAS NMR spectra of a series of octadecylsiloxane-bonded phases prepared by different reaction mechanisms and the silica substrate. 1 = Porous silica (structural elements Q -85 to -115 ppm); 2 = monomeric phase prepared with octadecyldimethylchlorosilane (M 15 to 0 ppm); 3 = polymeric phase prepared by the solution method with octadecylmethylchlorosilane (D -5 to -25 ppm); 4 = polymeric phase prepared by the solution method with octadecyltrichlorosilane (T -45 to -70 ppm); and 5 = surface polymerized phase using octadecyltrichlorosilane. (Adapted from ref. [110]. ©American Chemical Society).

silanol groups, the type of surface bonding of the organosilane, and to gain insight into the conformation of the bonded ligand in the dry and solvated states [61,63,107-110]. The large chemical shift dispersion of about 130 ppm for organosilanes and silica allows structural elements to be assigned quite easily from the ^{29}Si NMR spectra, Figure 4.7. FTIR and Raman spectroscopy can be used to study changes in the type and number of silanol groups, conformation and order of the bonded phase, and the presence of ligand functional groups [31,32,61,111,112]. Spectroscopic studies show that chain motion increases with distance from the surface, the chain mobility decreases with increasing surface coverage or after endcapping, and also decreases in the presence of water. Octadecylsiloxane-bonded phases formed from trifunctional

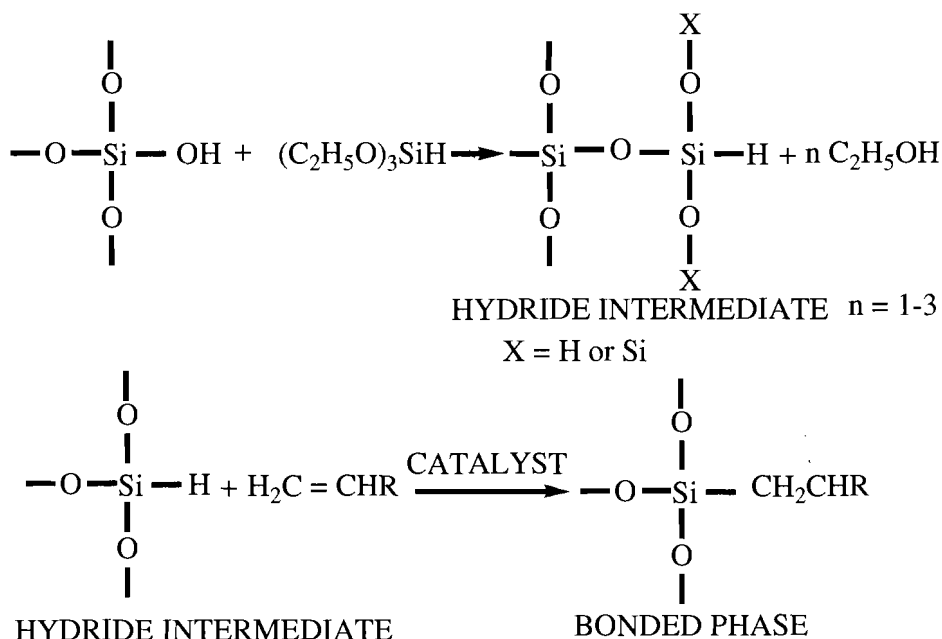


Figure 4.8. Two-step hydrosilanization and hydrosilation reaction for the formation of bonded phases on silica.

organosilanes show restricted motion in the region of the anchor group suggesting a brush-like or haystack-like structure. Octadecylsiloxane bonded phases prepared from monofunctional organosilanes can move freely suggesting a blanket structure with the top terminal part of the alkyl ligand bent to the surface of the silica substrate. Endcapping of these packings causes a change in order from a blanket structure to a brush or haystack configuration.

4.2.2.2 Hydrosilanization/hydrosilation bonded phases

The two-step hydrosilanization and hydrosilation reaction provides an alternative approach for the synthesis of bonded phases on silica and other inorganic oxides [66,80,113]. A hydride-modified silica intermediate is prepared by the controlled deposition and reaction of the acid hydrolysis products of triethoxysilane resulting in monolayer coverage of the silica with silane groups, Figure 4.8. In the second step, the silica-hydride intermediate is reacted with a terminal olefin (or possibly an alkyne [114]) in a suitable solvent containing a metal complex [66,113-115] or free radical catalyst [115,116]. The reaction proceeds through the formation of an intermediate olefin-metal complex of considerable size when the catalyst is hexachloroplatinic acid, the most widely used catalyst for the addition reaction. For steric reasons, therefore, not all silane groups can be reacted with the metal complex resulting in a bonding density that is lower

than the surface coverage of silane groups. In addition, complete removal of catalyst by-products, mainly platinum metal, from the bonded phase material is difficult and may be a problem for some chromatographic applications. The free radical initiation reaction with peroxides or azoisobutyronitrile was explored to solve both of these problems. Early results suggest some success, although reaction with the silica-hydride intermediate is accompanied by some polymerization of the olefin as a by-product. The hydrosilanization/hydrosilation reaction is little used to manufacture bonded phase materials at present, but it provides a versatile approach for the production of research quantities of materials, particularly bonded phases containing ligands too reactive for bonding by direct reaction with an organosilane.

4.2.3 Polymer Coated Inorganic Oxides

The driving force for the development of polymer coated particles is the wish to combine the favorable mechanical and morphological features of the porous inorganic oxides with the chemical inertness and variable selectivity of organic polymers by immobilizing an organic polymer within the inorganic oxide matrix [46,117-120]. For porous silica the coated polymer provides additional shielding of unreacted silanol groups and protects the silica matrix against chemical attack by aggressive mobile phases. For the metal oxides it overcomes the problem of a lack of a suitable mechanism for the preparation of chemically bonded phases and diminishes the problems associated with the strong adsorption of certain polar compounds by metal oxides. Polymer coating is an important technique for metal oxides, since there are no competing techniques to modify their properties. For porous silica the situation is different. Developments in siloxane-bonded phases have more than kept pace with those in polymer-coated phases, and siloxane-bonded phases remain more widely used than polymer-coated phases.

Polymer coated phases can be divided into two categories. Polymer coated or polymer encapsulated phases with a thin film of polymer extending over the surface of the inorganic oxide and polymer composites or gel-in-a-shell materials where the whole pore volume is filled with polymer. The polymer-coated phases are generally used for analytical separations where efficiency and speed are more important than capacity. Inorganic oxides can be modified by either surface coating or pore filling with polymer. For nearly all-practical applications further reactions between the polymer chains or between the polymer and the inorganic oxide are needed for stabilization of the polymer layer. This is most easily achieved by free radical crosslinking of the polymer chains or surface bonding between the polymer chains and either silanol groups or other surface anchored reactive functional groups introduced prior to coating. Crosslinking result in stable layers that are insoluble in common mobile phases. Formation of chemical bonds with the inorganic oxide surface is not essential, but it may improve the overall stability of the phase. Polymer composites are prepared by copolymerization of a monomer with an appropriate crosslinking agent or by crosslinking of a previously prepared polymer within the pores of the inorganic oxide [5,119,121-123]. The inorganic oxide provides a strong porous shell as a container for the soft hydrogel, which controls retention and

selectivity. These materials, known hyper-diffusive particles, are used for preparative separations of biopolymers (section 11.3.7.2).

Porous silica remains the most widely used substrate for the preparation of polymer-coated phases [119]. Deactivation of surface silanol groups by silanization [124,125] or reaction with a polyhydrosiloxane [126] prior to coating results in a final product with greater chromatographic inertness and improved coating properties for non-polar polymers. Deactivation and introduction of anchor groups for surface bonding is possible using reagents such as vinylchlorosilanes [127,128]. Immobilization of the physically adsorbed polymer layer is usually performed by free radical crosslinking initiated by organic peroxides or γ -radiation [119,124-131]. A variety of polymer can be immobilized in this way including poly(siloxanes), poly(butadiene), poly(styrene), various poly(acrylamides), poly(ethylene oxides), various polysaccharides, poly(vinyl pyrrolidone), poly(ethyleneimine), etc. [117-119]. Optimized immobilization conditions vary with the polymer but are generally quite straightforward. Typically, a mixture of the polymer and organic peroxide in a defined ratio is deposited from solution in the silica pores. The solvent is slowly evaporated and the coated particles heated to about 150°C for a few hours to complete the crosslinking reaction. Excess polymer is then removed by extraction. An alternative method uses the chemical vapor deposition of 1,3,5,7-tetramethylcyclotetrasiloxane to form a siloxane-hydride polymer film on the silica substrate. A wide range of functional groups (e.g. octadecyl, aminoalkyl, benzenesulfonic acid, etc.) can be introduced into the siloxane-hydride polymer film using the hydrosilation reaction [132,133]. High-surface area silica substrates allow materials with a high carbon loading (similar to conventional siloxane-bonded phases) to be synthesized by this approach.

The complex surface chemistry of the metal oxides (section 4.2.1.2) is incompatible with their use in a number of chromatographic techniques. Polymer coated metal oxides are seen as an important approach to extending their scope. Alumina and zirconia particles coated with poly(butadiene), poly(styrene), poly(ethylene oxide), a copolymer of chloromethylstyrene and diethoxymethylvinylsilane and succinylated poly(ethyleneimine), for example, have been prepared for use in reversed-phase, size-exclusion and ion-exchange chromatography [44,46,54,120,134-137]. The methods of preparation are similar to those used for porous silica.

Improved surface shielding properties and stability towards eluents of high pH are the main advantages of the polymer-coated phases. Surface silanization and polymer coating can minimize unfavorable interactions with silanol groups on porous silica or Lewis acid/base sites on metal oxides but cannot eliminate them entirely. In the case of non-polar polymer-coated zirconia access to about 50-75 % of the surface active sites can be blocked [137]. As well as imperfections in the polymer coverage the thin films essential for reasonable mass transfer properties (about 0.5-1.0 nm) must represent an imperfect diffusion barrier for the surface. Imperfect coverage is probably the reason why polymer-coated silica particles show improved high pH stability resulting in slower rather than limited dissolution of silica. Since zirconia is stable at all practical pH values, polymer-coated zirconia particles have exceptional pH and temperature stability.

Non-polar polymer-coated zirconia particles have been used with extreme pH mobile phases at temperatures above 150°C without affecting the retention properties of the sorbents [136-138]. The efficiency of polymer-coated inorganic oxides is acceptable but less than that of conventional siloxane-bonded phases. An increase in the contribution from resistance to mass transfer to the plate height related to two properties of the polymer film are responsible for this property [130,135,137-140]. Formation of highly crosslinked polymer films may be accompanied by the introduction of strongly retentive micropores in the polymer layer and preferential filling of the small pores by the polymer solution during the coating process can lead to blocking of constricted pores and slower mass transport. In addition, poor wetting of polar inorganic oxide surfaces by low polarity polymers could lead to a patchy film with regions of variable thickness or coverage.

4.2.4 Porous Polymers

Porous polymers have found increasing use for those applications that silica-based materials are inadequate due to their hydrolytic instability or surface heterogeneity [3-5,23,65,141-144]. The porous polymers are totally organic with a homogeneous surface devoid of strong bonding sites, and depending on their structure, can frequently be used with mobile phases of extreme pH where silica-based materials are degraded due to formation of soluble silicates or hydrolysis of siloxane-bonded ligands. They are often considered more suitable than silica-based bonded phases for the separation of inorganic ions, basic compounds and biopolymers that require relatively severe separation conditions using ion-exchange, reversed-phase or size-exclusion chromatography. The application range for some porous polymers is limited, however, by low compression resistance, inadequate dimensional stability towards different solvents and poor efficiency.

Macroporous polymers are generally prepared by a two-phase suspension reaction system consisting of vinyl monomers (e.g. styrene), in a mixture of water, organic solvent, crosslinking agent (e.g. divinylbenzene), initiator (e.g. benzoyl peroxide) and a suitable porogen [4,143-145]. Adjusting the stirring rate controls the droplet size in which the growing polymer chains form and eventually precipitate when they reach a critical size. The resultant particles are approximately spherical with a reproducible particle size range comprised of many hard microspheres interspersed with pores and channels, Figure 4.9. A suspension stabilizer (e.g. surfactant) may be required to prevent the droplets coalescing during polymerization. Poly(styrene-co-divinylbenzene) particles containing less than about 12% of crosslinking reagent by weight are soft and microporous. Rigid, macroporous particles suitable for high-pressure liquid chromatography contain greater than 20% (and often more than 50%) divinylbenzene. In terms of structure it should be noted that divinylbenzene is typically a technical mixture of 55% meta and para isomers, 42% ethylvinylbenzene and 3% diethylbenzene. The pore structure is adjusted by using various ratios of a porogen in the presence of a crosslinking agent, to form a rigid three-dimensional structure.

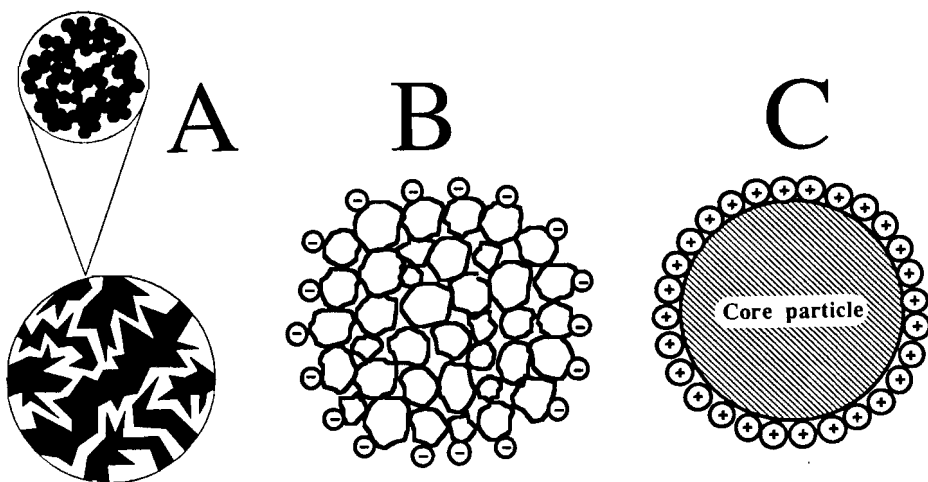


Figure 4.9. Different types of polymer particles for liquid chromatography. Macroporous polymer (A), surface functionalized porous polymer for cation-exchange chromatography (B) and electrostatically agglomerated anion-exchange particle (C).

The porogen is usually an inert solvent, or mixtures of inert solvent and polymers. The meso- and macropores in the polymer network are the voids once occupied by the porogen. Individual recipes for the preparation of macroporous polymer beads may seem complex in terms of the number of components involved and the required control of the experimental conditions. The technology, however, for their preparation has been developed to such a degree that excellent control over their properties (e.g. particle size, shape, porosity and chemistry) is routinely achieved. The vast majority of current macroporous polymers are based on styrene-divinylbenzene copolymers. Other suitable monomers include acrylates, methacrylates, hydroxyalkylacrylates, vinylpyridines and vinyl acetate. A wide range of products are available for HPLC in particle sizes from 5–20 μm , pore diameters from about 2–400 nm, and surface areas from about 50–500 m^2/g [141,144,146–148].

Truly monodisperse porous polymer particles can be prepared by a multi-step swelling and polymerization method utilizing a seed particle as a shape template [149–153]. A latex suspension of monodisperse seed particles, about 1- μm diameter, is activated by absorption of a water insoluble solvent. The enlarged seed particles, monomers, crosslinking agent, initiator, surfactant and porogen are stirred together in a suspension, transferring the reagents to the swollen seed particle, which is then subjected to suspension polymerization. In a common variation the enlarged seed particle is swollen with monomers, etc., and polymerized to create the porogen. These particles are then used to absorb the polymer reagents and subjected to suspension polymerization. After removal of the porogen by extraction, monodisperse beads with a well-controlled pore structure are obtained. Some unusual materials prepared

in this way include porous poly(vinylphenol-*co*-divinylbenzene) particles for size-exclusion chromatography [151] and porous poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) particles for normal-phase chromatography [152].

The separation of small molecules using porous polymers is characterized by modest efficiency and peak tailing for some solutes [142,144,148,154-158]. This is an inherent feature of the biporous particle structure. The polymer particles consist of meso- and macropores between the agglomerated microspheres together with micropores extending throughout the microspheres. The microporosity of the polymer matrix is formed by the interstices between the polymer chains. The poor chromatographic efficiency for small molecules results from their sorption into the polymer matrix (micropores) where they experience hindered diffusion. The column efficiency depends strongly on the sample type, solvent, and temperature. It generally decreases with increasing retention. In good solvents, such as moderately polar organic solvents and aqueous mixtures containing acetonitrile or tetrahydrofuran, the micropores are swollen and column properties are most predictable. The reduced hindered diffusion observed for these solvents is due to the solvent blocking a portion of the micropores and preventing sample from diffusing into them. In poor solvents the microspheres are less swollen, the column efficiency lower, and retention greater. The latter results from enhanced sorption due to the larger surface area contained within the micropores. Biopolymers are excluded from the micropores because of their size and therefore the difference in chromatographic efficiency between porous polymers and silica-based materials is less marked. When separating biopolymers it is essential for maximum efficiency that a sorbent with a sufficiently large nominal pore size is used to minimize zone broadening due to restricted diffusion within the porous matrix. This condition is satisfied if the ratio of the molecular diameter to the mean pore diameter exceeds 0.2.

The chromatographic properties of porous polymers can be easily changed by choosing different vinyl monomers or by chemical reaction of the preformed polymeric beads. A common modification is the introduction of ionic functional groups to produce packings for ion exchange and ion chromatography [3,4,143,159,160]. Nearly all commercially available materials contain a sulfonic acid group (strong cation exchanger), carboxylic group (weak cation exchanger), tetraalkylammonium group (strong anion exchanger) or an amine (weak anion exchanger) as the iononic functional group. Direct reaction of the aromatic rings of poly(styrene-*co*-divinylbenzene) materials with sulfuric or chlorosulfonic acid, or chloromethylation followed by amination, are widely exploited reactions for introducing sulfonic acid and tetraalkylammonium groups, respectively [4,143]. Poly(styrene-*co*-divinylbenzene) polymer materials containing one functional group per benzene ring would have an ion-exchange capacity of about 4.5 mequiv./g. Commercially available ion-exchange packings usually have capacities of about 2.5-4.0 mequiv./g. For life science applications ion exchangers have been prepared by adsorbing poly(ethyleneimine) onto the surface of a porous polymer and subsequently immobilizing the polymer film by crosslinking [144]. This avoids the strong non-specific adsorption of biopolymers observed on poly(styrene-*co*-divinylbenzene)

ion exchangers. A number of ion exchangers prepared from different surface-coated hydrophilic polymers on porous poly(styrene-*co*-divinylbenzene) are available.

Low-capacity ion exchangers, 10-100 $\mu\text{equiv./g}$, are generally used in ion chromatography to facilitate sample elution by eluents of low ionic strength with conductivity detection [159-164]. These materials are generally of two types: surface functionalized porous particles and electrostatically agglomerated pellicular particles, Figure 4.9. Surface functionalized materials can be prepared from microporous or macroporous polymers by conventional techniques, modified to produce a low degree of functionalization. For example, a macroporous poly(styrene-*co*-divinylbenzene) bead immersed in concentrated sulfuric acid for less than 30 s will give a material in which the sulfonic acid groups are confined to a very shallow depth (of the order of 20 nm) around the outside of the particle. Modern materials with optimized selectivity for specific separations are generally prepared by grafting a thin film (1-10 nm) of ion exchange polymer on a base poly(ethylvinylbenzene-*co*-divinylbenzene) or methacrylate substrate [165-169]. Highly crosslinked particles provide a rigid support with a controllable surface area compatible with organic and aqueous solvents. The grafted polymer layer provides control over the concentration and type of ionic groups and facilitates the preparation of materials with mixed ionic groups.

Historically, electrostatically agglomerated pellicular materials have been the most widely used for the popular suppressed ion chromatography separation mode (section 4.3.7). These materials consist of a monolayer of charged latex particles, which are electrostatically attached to a surface functionalized core particle, Figure 4.9. The core particle is usually a poly(styrene-*co*-divinylbenzene) bead of moderate crosslinking, about 5 to 15 μm in diameter, which has been functionalized to carry a charge opposite to that of the latex particles. The latex particles are much smaller, about 10-500 nm, and are fully functionalized to contain the desired ion exchange group. The latex layer is very stable and is not effected by normal column operating conditions and mobile phases. The latex particles are prepared from a base monomer to create the ion exchange site, a crosslinking monomer to control water content and possibly a non-functional monomer to adjust the charge density or control secondary interactions that influence selectivity. Styrene is commonly utilized as the base monomer for the preparation of cation exchange materials and vinylbenzyl chloride or glycidylmethacrylate for anion exchange materials. Divinylbenzene is usually used for crosslinking aromatic monomers and ethylene glycol dimethacrylate for methacrylate monomers. Typical latex ion exchange groups include alkanolamines, quaternary amines, sulfonic acids, carboxylic acids and phosphonic acids. Since the selectivity of the material depends primarily on the type of ionic group and structure of the ion exchange site, a large number of materials have been developed to facilitate specific separations. The electrostatically agglomerated particles create an ion exchange pellicular layer within which the ion-exchange separation takes place and an oppositely charged underlayer, which excludes ions of similar charge through Donnan potential forces. The high permeability and fast kinetics of the latex layer and the small particle size promotes high chromatographic efficiency.

4.2.5 Porous Graphitic Carbon

Initial studies of active and graphitized carbons, similar to those used successfully in gas chromatography (section 2.3.7.2), produced disappointing results when applied to liquid chromatography. These materials exhibited poor mechanical strength and poor peak shapes resulting from strong interactions within micropores and with (mainly) mineral and oxygen- and nitrogen-containing surface impurities of high activity. A breakthrough was achieved with the development of the template replication method that allowed rigid, mesoporous graphitic carbon particles to be produced in a reproducible manner [23,170-172]. The production of this material is complex and catalyzed interest in other lower-cost materials. Notable among these are carbon-clad porous zirconia particles prepared by chemical vapor deposition methods [173,174] and glassy carbon coated porous silica and zirconia particles prepared by low-temperature graphitization of precoated polymers [175]. However, only porous graphitic carbon (Hypercarb®) and carbon-clad zirconia are commercially available.

Porous graphitic carbon is synthesized by a multistep chemical and thermal treatment from organic monomers deposited in the pores of a silica gel particle template and subsequently subjected to polymerization, carbonization, dissolution of the silica template and graphitization [170,172]. The silica gel template allows optimization of the adsorbent particle size, porosity and surface area for liquid chromatography. The selection of monomers and thermal treatment is responsible for the mechanical strength, high purity and absence of significant microporosity. Commercially available materials have particle sizes of 5 or 7 μm , a mean pore diameter of 25 nm and a surface area of 100-120 m^2/g .

Porous graphitic carbon is composed of a network of intertwining graphite ribbons held together with covalent bonds resulting in a mechanically strong structure with better defined surface properties and minimization of surface defects associated with heteroatom active sites. The extensive network of overlapping hybridized carbon orbitals results in a surface that is polarizable and a suitable partner for electron lone pair donor-acceptor interactions. The exceptional separation of stereoisomers, diastereomers and polychlorinated biphenyl congeners is believed to result from the planarity of the surface and the closeness of the fit between isomers and the graphite surface [170,176-178]. Since the graphite surface is chemically unreactive it cannot be modified directly. Ion exchange and enantiomer selective phases, however, have been prepared by the surface adsorption of suitable polymers [53,179,180]. The high heats of adsorption of polymers on porous graphitic carbon result in coated materials of exceptional stability.

For compounds of a varied structure, there is little correlation between the reversed-phase retention order observed on common chemically bonded phases and porous graphitic carbon [170,172,181]. Porous graphitic carbon behaves as a strong hydrophobic sorbent with increased retention compared to typical silica-based octadecylsiloxane-bonded materials. Strong electron lone pair and dipole-type interactions between the graphite surface and analyte functional groups account for the additional retention of polar compounds [178]. These polar interactions probably depend on the orientation of the polar functional groups to the graphite surface, and therefore, analyte structure.

Hydrogen-bonding interactions are more favorable in the mobile phase resulting in lower retention. One manifestation of the importance of polar interactions is the significant retention of low-molecular-mass ions and the effect of competing ions on selectivity [182,183]. For similar separation conditions, these ions are generally unretained by chemically bonded phases. In addition, porous graphitic carbon is eminently suitable for the separation of compounds requiring extreme pH conditions.

4.2.6 Nonporous Particles

Nonporous silica and polymeric microparticles with diameters of 1-5 μm were introduced in the 1980s for the fast separation of biopolymers [184-186]. The surface heterogeneity and tortuous pore structure of totally porous microparticles is responsible for additional peak broadening and occasional loss of biological activity for biopolymers. Nonporous microparticles are devoid of a pore structure providing rapid mass transfer between the mobile and stationary phases and have a low surface area (0.5-3 m^2/g) with a reduced denaturing capacity. By virtue of their favorable mass transfer properties columns can be operated at high mobile phase velocities ($u > u_{\text{opt}}$) without a serious loss in efficiency. On the other hand, the small particle size creates a high-pressure drop, which limits column length (usually < 3.5 cm) and the ultimate mobile phase velocity that can be employed. Above ambient temperatures are often used to reduce the mobile phase viscosity and enhance the separation speed. The lack of an internal pore structure also facilitates rapid column equilibration in gradient elution commonly used for the separation of biopolymers. The low sample capacity of nonporous particles (which is effectively similar to conventional wide pore porous particles for biopolymers) requires a careful choice of sample injection volume and technique [14,184,187]. For fast separations the small interparticle column volumes (usually < 250 μl) require that extracolumn volumes are minimized and fast signal processing conditions are used. This may require modification of instruments designed for use with conventional columns. The low surface areas of nonporous particles provide inadequate retention of small molecules, and there are few applications to their separation [188,189].

Nonporous silica particles are usually prepared by the hydrolysis of a tetraalkoxysilane in a mixture of aqueous ammonia and ethanol to form a monodisperse colloidal silica dispersion with particle sizes up to 0.7 μm [14,36]. The particle size depends on the ammonia concentration, the composition of the ethanol solution, the identity of the tetraalkoxysilane and the reaction time. The colloidal silica particles are subjected to a layer-by-layer controlled growth phase to achieve the larger particle sizes useful for liquid chromatography. Calcining at 1000°C is used to remove micropores that may have formed during the synthesis. Nonporous polymer particles are prepared by adaptation of the suspension polymerization and multistep swelling and polymerization methods (section 4.2.4), or by dispersion polymerization [185]. Surface modification of nonporous particles by chemical reaction or polymer coating uses the same techniques employed for porous particles.

4.2.7 Monoliths

Monoliths (or continuous bed columns) are a new concept in column technology, which apart from particle-loaded monolithic sol-gel columns, do not depend on particle technology for their operating characteristics [190-194]. A monolith is a unitary porous structure formed by *in situ* polymerization or consolidation in a mold (often the column). Four general approaches have been used to synthesize monoliths: (1) copolymerization of water-soluble monomers (e.g. acrylamides) in the presence of an inorganic salt to form a continuous bed consisting of bundles of polymer chains with inter-bundle channels that permit eluent flow [193]; (2) copolymerization of organic monomers (e.g. styrene and divinylbenzene) in the presence of a porogen to form a continuous bed with a rigid macroporous structure [145,194,195]; (3) silica rods with a bimodal pore structure prepared by the hydrolysis and polycondensation of alkoxysilanes in the presence of water soluble polymers [196-200]; and (4) particle-loaded monoliths prepared by polymerization of a suspension of silica particles in the presence of a silica sol to form a continuous bed of particles held in place by an irregular silica skeleton with large throughpores [201]. All monoliths contain macropores to provide convective transport through the continuous bed. The interparticle voids in particle-packed columns account for virtually all the mobile phase flow through the column with mobile phase contained in the porous particles essentially stagnant. The slow diffusion of solutes, particularly biopolymers, between the streaming mobile phase and stationary phase is the main cause of poor mass transfer that limits separation speed. Convective transport allows an increase in the rate of mass transfer but its implementation in particle beds is limited by the presence of interparticle voids that provide an alternative flow path of low resistance compared with particle macropores. Employing a continuous structure with convective pores responsible for mobile phase flow connected to mesopores to control the surface area for stationary phase interactions circumvents this problem.

Polymeric monoliths with a low surface area have generally been used for the separation of biopolymers where a large surface area is not required. Monoliths with a wide range of pore sizes can be prepared by controlling temperature, concentration of crosslinking monomer and the use of a mixture of porogens [194,195]. Functionalization, when required for different chromatographic modes, is achieved either by modification of the polymer surface by direct reaction, or by grafting with suitable functional monomers at the pore surface.

The silica monoliths with a bimodal pore structure are prepared by a sol-gel method from alkoxysilanes in the presence of a water-soluble organic polymer. For example, to an acetic acid solution of poly(ethylene oxide), molecular weight about 10,000, is added tetramethoxysilane as a source of silica. The solution is homogenized and poured into a mold of appropriate size and thermostatted at 40°C to promote phase separation and gelation. After aging the silica rods are subjected to a controlled pore redistribution process using aqueous ammonia at elevated temperatures. After drying the monoliths are strengthened by heating to 700°C. The silica surface can be modified using conventional organosilane reagents for use in different separation modes. Monolithic

silica rod and polymer columns for analytical and preparative liquid chromatography were introduced commercially around 1999.

Pore network modeling theory indicates that monolithic structures of acceptable chromatographic efficiency with a low-pressure drop should have relatively large throughpores with high pore connectivity [202-204]. The bed structure should have small sized skeletons with mesopores of an appropriate pore size distribution (an average pore size relatively large compared to the size of the diffusing solute) and high pore connectivity. This is largely born out by plots of the plate height and column pressure drop against mobile phase velocity for octadecylsiloxane-bonded silica monoliths [190,198,204-206]. Commercially available silica rod columns (10 cm x 4.6 mm I. D.) have throughpores of 1.5-2 μm , diffusion pores about 12 nm, a specific surface area of 300-350 m^2/g , and a total porosity of 0.87 (porosity of throughpores 0.69 and mesopores 0.18). They produce a minimum plate height of about 10-15 μm , in the same range as a conventional particle-packed column containing 5 μm particles, at a 2-3 times higher mobile phase velocity. The column pressure drop depends largely on the size of the macropores. For various materials with useful separation properties it is generally somewhere between 2-10 times lower. The lower pressure drop and favorable mass transfer properties at high flow rates suggests that these columns should be more suitable for fast separations than particle-packed columns.

4.3 RETENTION MECHANISMS

Retention in liquid chromatography is a complex process involving solute interactions in both the mobile and stationary phases that are difficult to describe exactly. This situation mirrors our poor fundamental knowledge of solution chemistry for all but simple systems, which chromatographic phases are not. Attempts to describe retention can be divided into two categories. Simple and sometimes empirical models based on readily accessible information that enable reasonable estimates of retention to be made but provide a poor and in some cases an erroneous description of the separation process. Rigorous models grounded in thermodynamic concepts that attempt to accurately describe the separation process but are difficult to use in practice. The problem here is a lack of knowledge (or easy method of determination) of molecular parameters incorporated in the models and the complex calculation procedures required. In a few cases it could be stated that contemporary knowledge represents a middle ground between the two extremes.

The approach adopted here is to emphasize mechanistic information at a level useful for an understanding of the retention process and for qualitative estimation of retention, where this seems appropriate. A rigorous discussion of theoretical models that have limited practical utility is left to the cited references. From the perspective of practical utility the sometimes totally empirical or statistical models presented in the method development section (section 4.4) are a further source of information on how to obtain separations with a limited knowledge of mechanistic details.

4.3.1 Reversed-Phase Chromatography

Reversed-phase liquid chromatography (RPC) is the most popular of the liquid chromatographic methods based on its exceptional range of applications, versatility and simplicity of operation [61,207,208]. The simultaneous separation of neutral and ionic solutes is possible and the rapid equilibration of the stationary phase with changes in the mobile phase facilitates the use of gradient elution methods. The addition of additives to the mobile phase allows exploitation of secondary chemical equilibria, such as ion suppression, ion pair formation, metal complexation, and micelle formation to augment selectivity changes from varying the mobile phase composition. Since the mobile phase is generally an aqueous solution, RPC is ideally suited to the separation of polar molecules which are either insoluble in organic solvents or bind too strongly to inorganic oxide adsorbents for normal-phase chromatography. Many biological polymers fall into this category and the development of wide pore reversed-phase packings has had a profound effect on the separation of biopolymers in the life sciences and biotechnology [23,184,209-212]. RPC employing acidic, low ionic strength eluents has become a widely established technique for the purification and structural elucidation of biopolymers. The structure of biopolymers, however, is very sensitive to mobile phase composition, pH, temperature, ionic strength and stationary phase type [211-216]. Multisite interactions with the stationary phase can result in anomalous retention behavior and peak splitting if the stationary phase topography and that of the biopolymer are incompatible. Multisite interactions, which require additional time for the conformation of the molecule to attain the right position on the stationary phase surface, also result in much slower kinetics for the adsorption and desorption process, and lower column efficiency than is generally observed for low molecular weight solutes.

4.3.1.1 General Consideration

There are several hallmarks of a separation by reversed-phase liquid chromatography that distinguishes them from other liquid chromatographic methods. Often a broad definition of reversed-phase separations is used to include all separations that employ a polar mobile phase and a relatively less polar stationary phase. This definition includes, but fails to emphasize, a key feature of most practical applications of reversed-phase chromatography. This is that the mobile phase is an aqueous solution, and the presence of water plays an important role in establishing the hallmark features of these separations. The high cohesive energy, hydrogen-bond acidity and dipolarity of water are responsible in large part for the general increase in retention with increasing solute size and reduced retention of polar solutes and ions, which establish favorable intermolecular interactions with water. A decrease in polarity of the mobile phase, equivalent to increasing the volume fraction of organic solvent in an aqueous mixture, leads to a decrease in retention; a reversal of the general trends observed in liquid-solid or "normal-phase" chromatography. This explains the origin of the name reversed-phase chromatography but is only of historical interest today. For binary mixtures of

an organic solvent in water the change in solute retention factors with mobile phase composition in reversed-phase chromatography can be described by Eq. (4.2) [217-224]. Over a narrow composition range Eq. (4.2) can often be simplified to the linear relationship described by Eq. (4.3).

$$\log k = \log k_w + a\phi + b\phi^2 \quad (4.2)$$

$$\log k = \log k_w - S\phi \quad (4.3)$$

In the above equations k is the solute retention factor, k_w the solute retention factor for water as the mobile phase, ϕ the volume fraction of organic solvent, S the slope of the experimental data after fitting to a linear regression model, and a and b are regression coefficients obtained by fitting the experimental data to a second order polynomial model. Assuming that the composition and volume of the stationary phase is unaffected by the change in composition of the mobile phase, only a poor approximation at best, then S in Eq. (4.3) is equivalent to the free energy of transfer of the solute from water to the organic solvent and should be independent of the identity of the stationary phase. These considerations led to the general use of the S -value as a measure of the solvent strength of the organic solvent (section 4.4.1.1). S -values are widely used in method development to select binary mobile phase compositions of the same strength but different selectivity. S -values, although often similar for low molecular weight solutes, do vary with structure, tending to increase with solute size and decreasing polarity. S -values, therefore, should be considered as only semi-quantitative descriptors of solvent strength.

In practice, plots of $\log k$ against volume fraction of organic solvent are often curved when the volume fraction of organic solvent at either extreme of the composition range ($\phi \rightarrow 0$ or $\phi \rightarrow 1$) is included in the plot, Figure 4.10 [158,220,225]. For individual solutes linear, convex or concave plots are observed for the same system. Across different systems the shape of the plots often change for the same solute. For the intermediate mobile phase composition range, an approximate linear relationship based on Eq. (4.3) can almost always be found, but the intercept obtained by linear extrapolation is generally not identical to the intercept found by curve fitting the full range of experimental data, Table 4.4. Also, the intercept obtained by linear extrapolation is usually dependent on the identity of the organic modifier. The direct determination of $\log k_w$ is difficult and time consuming on account of the low solvent strength of water, but where reliable measurements are available there is only a poor correlation with the values obtained by linear extrapolation based on Eq. (4.3). Taken together, the above observations indicate that it is unlikely that the use of Eq. (4.3) results in an accurate estimate of $\log k_w$, in spite of frequent contrary statements in the literature. The widespread use of $\log k_w$ obtained by extrapolation as a general scale of solute hydrophobicity or as a solute descriptor in correlation models of biopartitioning should be treated as circumspect [220,226,227]. The value of $\log k_w$ obtained by extrapolation probably has some thermodynamic significance, as discussed elsewhere

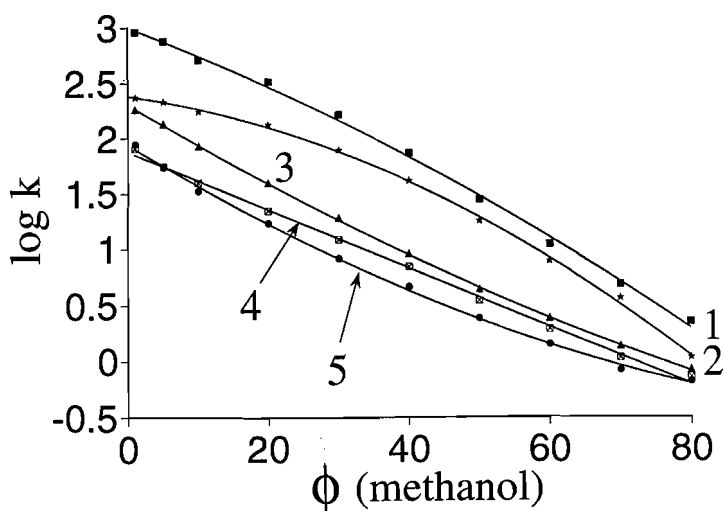


Figure 4.10. Plot of the retention factor as a function of the volume fraction (% v/v) of organic solvent in reversed-phase chromatography. Stationary phase is an octadecylsiloxane-bonded silica sorbent with methanol-water as the mobile phase. Solute identification: 1 = naphthalene; 2 = bromobenzene; 3 = acetophenone; 4 = 2-phenylethanol; and 5 = benzamide.

Table 4.4

Comparison of experimental and extrapolated $\log k_W$ values for an octadecylsiloxane-bonded silica sorbent

Solute	Organic Solvent	$\log k_W$ Quadratic ¹ Eq. (4.2)	Linear ² Eq. (4.3)	Experimental
2-Phenylethanol	Methanol	2.36	2.00	2.45
	Acetonitrile	2.05	1.27	
	Tetrahydrofuran	1.65	1.32	
Acetanilide	Methanol	2.19	1.60	2.52
	Acetonitrile	1.92	1.03	
	Tetrahydrofuran	1.39	1.04	
Acetophenone	Methanol	2.81	2.26	3.01
	Acetonitrile	2.33	1.71	
	Tetrahydrofuran	1.99	1.59	
Benzaldehyde	Methanol	2.43	1.87	2.56
	Acetonitrile	1.94	1.66	
	Tetrahydrofuran	1.74	1.56	

¹From 1 to 100% (v/v) methanol and acetonitrile and 1 to 70% (v/v) tetrahydrofuran.

²From 40 to 70% (v/v) methanol and acetonitrile and 30 to 60% (v/v) tetrahydrofuran.

[220,225,228]. It is unlikely, however, to be established by the distribution constant for the transfer of the solute between water and stationary phase solvated only by water.

A number of reports discuss the correlation between the slope and intercept derived from Eq. (4.3) of the form $S = p(\log k_W) + q$, where p and q are regression constants

[218-220,229]. Theoretically, a linear correlation between S and $\log k_w$ is expected for any system where a single solute interaction dominates retention. Members of a homologous series, for example, have similar polarity and differ only in size and should conform to the linear model. For compounds that differ significantly in the type and capacity for polar interactions only poor correlations are generally observed.

For members of a homologous series it is generally observed that the logarithm of the solute retention factor in reversed-phase chromatography is a linear function of the number of carbon atoms (methylene groups) of the structure, with the possible exception of the first few members of the series, Eq. (4.4) [223,230-233]

$$\log k = p n_C + q \quad (4.4)$$

where n_C is the number of methylene groups and p and q are regression constants. The slope of this plot is the logarithm of the methylene group selectivity and is a measure of the free energy of transfer of one methylene group from the mobile phase to the stationary phase. A small discontinuity in the plots is observed for monomeric phases with a high bonding density at the point where the sample chain length exceeds the bonded phase chain length. For solutes with long chains, the complete insertion of the solute molecules between the bonded chains is no longer possible. One portion of the solute chain penetrates inside the interphase region while the remaining solute methylene groups undergo weaker dispersion interactions at the interface between the interphase region and the mobile phase. It is also generally observed that branched chain compounds are retained to a lesser extent than their straight chain analogs and that unsaturated compounds are eluted before their fully saturated analogs.

The general influence of temperature on retention is described by the van't Hoff relationship discussed in section (1.4.4). Temperature provides a complementary optimization parameter to mobile phase composition for method development in reversed-phase chromatography. Temperature is an important variable in the separation of conformationally constrained compounds such as stereoisomers. Reversed-phase chromatography with aqueous or non-aqueous mobile phases is used for the shape-dependent separation of polycyclic aromatic compounds, steroids and carotenoids [73,75,234-236]. Primarily the type, bonding density, alkyl chain length and temperature influence shape selectivity on silica-based, chemically bonded phases. Mobile phase composition is less important for shape-selective separations but is constrained to provide sufficient solvent strength for convenient elution. Useful shape selectivity is observed for polymeric bonded phases with a high bonding density ($> 4 \mu\text{mol}/\text{m}^2$) at normal or low temperatures on wide pore silica substrates ($> 15 \text{ nm}$). A slot model was proposed to explain the elution order of isomeric polycyclic aromatic compounds, the retention of which was shown to increase in approximate order of their length-to-breadth ratio and planarity on polymeric octadecylsiloxane-bonded silica phases. For the separation of long chain carotenoids, retinoids and tocopherols polymeric alkylsiloxane-bonded silica phases with a C-30 alkyl chain provide improved isomer separations [235,236]. The shape selectivity of these phases correlates with the ratio of the more ordered trans con-

formation to the more flexible gauche conformation of the bonded alkyl chains. This ratio increases with higher bonding density and lower temperatures.

4.3.1.2 System Considerations

The strong intermolecular interactions between water molecules tend to promote self-association over interactions with dissimilar solvent or solute molecules. This preference of water to reform water-water interactions assists in the transfer of solutes to the stationary phase contributing to the driving force for retention in reversed-phase chromatography. As a consequence water is a weak solvent for reversed-phase separations. In addition, the same preference for self-association restricts the number and type of organic solvents that are miscible with water. For reversed-phase chromatography with UV detection methanol, 2-propanol, acetonitrile and tetrahydrofuran account for nearly all applications using binary mobile phases and most applications using ternary and higher order solvent combinations. On the one hand the limited number of miscible solvents simplifies method development approaches by minimizing the number of trial separations with different solvents. On the other hand, though, the dimensions of the selectivity space that can be explored are restricted to the narrow range of solvent properties provided by the water miscible solvents. At the microscopic level binary mixtures of water and organic solvents are heterogeneous containing clusters of associated water molecules, water-organic solvent aggregates, and associated organic solvent molecules with an equilibrium composition that depends on the volume fraction of organic solvent and its identity [237-242]. Although the existence of solvent clusters is well established, the absolute relationship between the bulk solution composition and that of the size and composition of the clusters is still debatable. For example, methanol-water mixtures are said to consist of clusters of water, methanol and methanol(water)₂ [237], while in another report the composition was indicated as methanol, water, methanol(water) and methanol(water)₅ clusters [238]. An inexact plot of the change in relative composition of methanol-water mixtures as a function of the volume fraction of methanol is shown in Figure 4.11. At low methanol concentrations the solution contains mainly water and water-methanol clusters. At the midpoint the solution contains mainly water-methanol clusters and lesser amounts of free and self-associated water and methanol clusters. At low water concentrations the solution contains mainly methanol and water-methanol clusters with a small fraction of free water. The formation of clusters with acetonitrile and tetrahydrofuran shows similar trends but the relative fraction of water-solvent complexes is significantly lower and the number and composition of these clusters more varied. In terms of retention it is assumed that solutes distribute themselves selectively between the stationary phase and individual solvent clusters characterized by different distribution constants. The overall solute distribution constant is an average of these different preferences.

The stationary phase in reversed-phase chromatography is poorly defined with a fluid structure, composition and volume that depends on the equilibrium mobile phase composition, the identity and bonding density of surface-restrained ligands, the number and type of accessible silanol groups, and temperature [108,109,240,243-245].

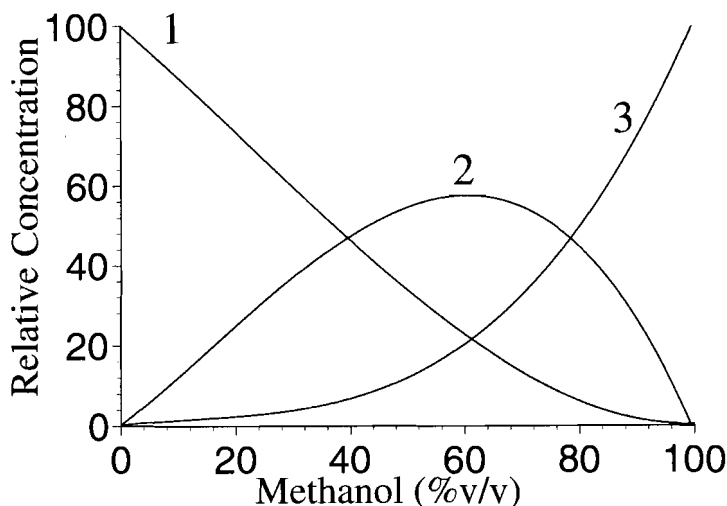


Figure 4.11. Symbolic plot of the relative concentration of water, methanol and methanol(water) clusters in a binary methanol-water mixture as a function of the volume fraction of methanol. Identification: 1 = water; 2 = methanol(water) clusters; and 3 = methanol.

247]. The selective sorption of mobile phase components and its influence on chain conformation results in a film of associated solvent molecules and surface-restrained ligands with a thickness and composition that changes with solvent type and mobile phase composition. In predominantly aqueous solutions the surface-restrained ligands are most likely in a collapsed state with intercalated solvent trapped within their structure. Access to surface silanol groups may be sterically restricted and the thickness of the stationary phase layer minimized. In predominantly organic mobile phases the surface-restrained ligands will be solvated and fully extended perpendicular to the surface. Mobile phase components will have greater access to the layer with penetration of solvent restricted by the ligand bonding density and the strength of ligand-ligand interactions. Water molecules are likely to be preferentially hydrogen-bonded to silanol groups and organic solvent molecules to the organic ligands. A gradient of solvent composition from the silica surface to the bulk mobile phase is possible with the organic solvent preferentially localized at the chain ends most distant from the surface. Solvents with a preference for hydrogen-bond interactions, such as methanol, are likely to drag associated water molecules with them into the stationary phase. This fluid stationary phase structure is sometimes referred to as an interphase with a permanent boundary provided by the surface of the silica substrate and a fluctuating boundary as an imaginary plane projected above the silica surface beyond which resides the mobile phase. The solvation properties of the interphase and the phase ratio of the system depend on the composition of the mobile phase and the structure of the stationary phase.

The selective uptake of mobile phase components by alkanesiloxane-bonded silica sorbents has been quantitatively determined by the thermodynamic sorption excess

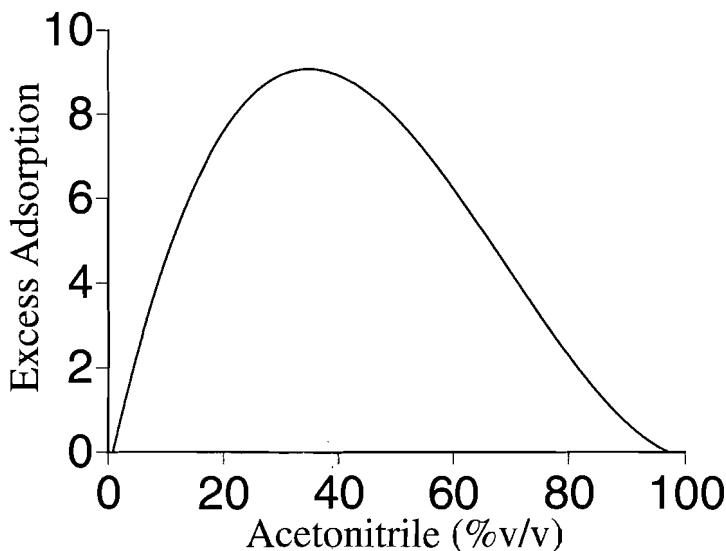


Figure 4.12. A typical plot of the excess adsorption ($\mu\text{mol}/\text{m}^2$) of acetonitrile by an octadecylsiloxane-bonded sorbent from a water-acetonitrile mobile phase.

quantity of the organic solvent with respect to the bulk mobile phase composition, Figure 4.12 [248-252]. These studies indicate a higher sorption excess for tetrahydrofuran compared with acetonitrile, which in turn, is greater than for methanol. When the incorporation of water into the solvation layer is considered, acetonitrile solvated layers contain the lowest amount of water followed by methanol, and the largest amount is observed for tetrahydrofuran, in broad agreement with expectations from consideration of the microheterogeneity of binary aqueous solutions. The results may be somewhat different for short alkanesiloxane-bonded chains (e.g. butyl groups) due to the easy access of mobile phase components to surface silanol groups [251].

Silanol groups participate in the retention process as adsorption and ion exchange sites and through their influence on the selective solvation of the interphase region. Even exhaustively derivatized silica surfaces contain a significant number of unreacted silanol groups whose accessibility to solutes in the mobile phase is governed by their local stereochemistry and selective solvation. Although normal silanol groups are weak acids ($\text{pK}_a = 5-7$), a small fraction, perhaps activated by metal impurities embedded in the silica substrate, demonstrate much higher acidity. These groups are important in the separation of basic compounds. As well as contributing to the general retention mechanism, they are the primary source of the often-observed poor chromatographic properties of these compounds, namely, tailing, irreversible adsorption and the strong dependence of retention on sample size [13,37,253-256]. A large number of bonded phase sorbents employing different bonding chemistry were introduced in recent years with the aim of minimizing these interactions (section 4.2.2.1).

It should be noted that all the above interactions take place within the porous structure of the silica substrate, which might further favor certain interactions because of local confinement and additional interactions between surface-restrained ligands located at distant positions on the curved walls of the confining pores. Also, the alkanesiloxane-bonded chains are at a relatively low packing density and entropically confined compared to a bulk liquid and therefore solvation models assuming bulk liquid properties for the stationary phase should be interpreted with care [257].

Conceptual models of the structure of solvated porous polymer and graphitic carbon stationary phases are not as well developed. Porous polymer sorbents of the type used in liquid chromatography are meso- and macroporous particles constructed from an agglomeration of extensively fused microspheres, which are themselves microporous materials (section 4.2.4). Reversed-phase retention on porous polymers is presumed to occur through solute interactions with the solvated surface of the polymer matrix lining the meso- and macropores, as well as with solvent imbibed by the micropores of the solvent-swollen matrix [142,157,158]. The selective uptake of mobile phase components by the micropores has a significant influence on the overall retention and selectivity of the separation, but in a manner that is incompletely understood for the present. In the case of porous graphitic carbon an adsorption mechanism is generally presumed with solvent effects controlling retention through mobile phase interactions (section 4.2.5) [170,178,181].

4.3.1.3 Solvation Parameter Model

The solvation parameter model has steadily gained acceptance as a general tool to establish the contribution of individual intermolecular interactions to the retention mechanism of neutral compounds in reversed-phase chromatography [221,258]. A general discussion of the solvation parameter model is provided in (section 1.4.3) and should be read first to follow the material in this section. The free energy of solute transfer from the mobile phase to the stationary phase is modeled as a series of product terms representing the solute contribution (solute descriptors) and chromatographic properties (system constants), Eq. (1.6 or 1.6a). These terms account for cavity formation, dispersion interactions, electron lone pair interactions, interactions of a dipole-type and hydrogen-bond acid and base interactions. The applicable form of the hydrogen-bond basicity solute descriptor for reversed-phase chromatography is $\Sigma\beta_2^0$. The system constants (m , r , s , a , and b) describe the difference in defined interactions and processes for the stationary phase (interphase region) and mobile phase. The m system constant characterizes the difference in free energy for cavity formation (cohesion) and dispersion interactions in the solvated stationary phase and mobile phase. The r system constant represents the difference in lone pair π - and n -electron interactions between the solvated stationary phase and mobile phase. The s constant represents the difference in solute dipole-dipole and dipole-induced dipole interactions in the solvated stationary phase and mobile phase. The a system constant is a measure of the difference in hydrogen-bond basicity and the b system constant the difference in hydrogen-bond acidity of the solvated stationary phase and mobile phase.

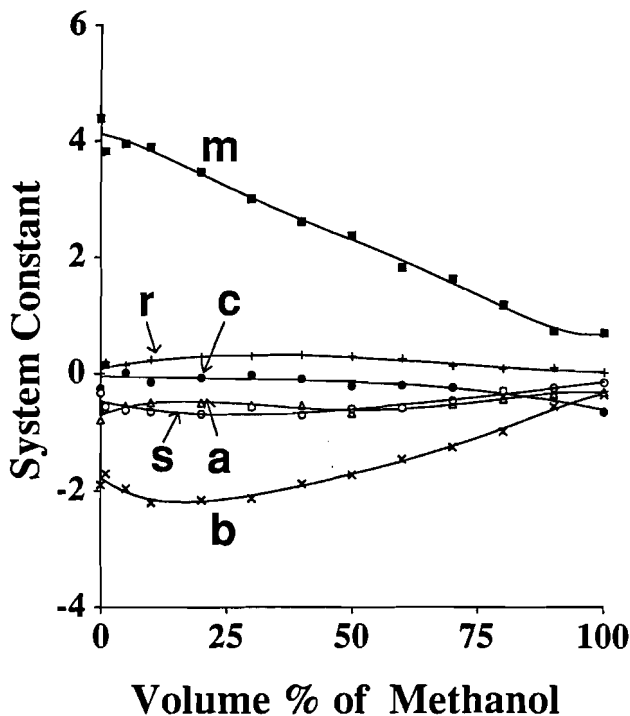


Figure 4.13. System map for an octadecylsiloxane-bonded silica sorbent with methanol-water mixtures as mobile phase.

Most studies in reversed-phase chromatography attempt to establish a relationship between the system constants of the solvation parameter model and properties of either the mobile or solvated stationary phases. The most useful representation of these relationships is a system map, Figure 4.13 [158,178,221,258-261]. System constants with a positive sign indicate that the defined property is more favorable in the solvated stationary phase than in the mobile phase and contributes to retention (the corollary is true for system constants with a negative sign). For chemically bonded sorbents the dominant contribution to retention in reversed-phase chromatography is the cavity and dispersion interaction term (m constant) sometimes with a small contribution from electron lone pair interactions (r constant). As the volume fraction of organic solvent increases the m constant decreases. This is indicative of a smaller difference in the cohesion and dispersion interactions between the mobile and solvated stationary phases. It is the basis of the observation that increasing the volume fraction of organic solvent in the mobile phase generally results in reduced retention in reversed-phase chromatography. Polar interactions are generally more favorable in the mobile phase and reduce retention (system constants have a negative sign). The dominant property of the mobile phase besides cohesion is its hydrogen-bond acidity. Since water is the

Table 4.5

Influence of solvent type on the system constants of the solvation parameter model for a cyanopropylsiloxane-bonded silica sorbent in reversed-phase chromatography ($s = 0$ in all cases)

Solvent	Volume fraction (% v/v)	System constant			
		m	r	a	b
Methanol	50	0.84	0.21	-0.20	-0.88
	40	1.09	0.24	-0.22	-1.15
	30	1.45	0.32	-0.24	-1.36
2-Propanol	50	0	0.15	-0.27	-0.10
	40	0.29	0.16	-0.27	-0.41
	30	0.84	0.20	-0.29	-1.05
Acetonitrile	50	0.40	0.05	-0.18	-0.54
	40	0.64	0.09	-0.21	-0.80
	30	0.98	0.15	-0.24	-1.06
Tetrahydrofuran	50	0.47	0	-0.11	-0.67
	40	0.70	0	-0.06	-0.93
	30	1.18	0	0	-1.45

most cohesive and hydrogen-bond acidic of the common solvents used in reversed-phase chromatography the dominant trends in Figure 4.13 illustrate the pivotal role of water in the retention mechanism. For binary mobile phases containing organic solvents other than methanol the same general trends are observed with numerical differences in the system constants representing the ability of these solvents to selectively modify system properties, Table 4.5 [262].

A number of studies report stationary phase properties at a single mobile phase composition or narrow range of compositions [258,263-268]. These studies are somewhat difficult to summarize in a global sense, since the mobile phase type or composition are often different. Most data are available for the mobile phase methanol-water (50:50), Table 4.6, and acetonitrile-water (30:70) [258]. Individual system constants vary widely with the identity of the stationary phase. Selectivity differences between phases, however, are preferably correlated through differences in the system constant ratios (r/m , s/m , a/m and b/m) [267-269]. There must be a significant difference in at least two system constant ratio for effective changes in band spacing on any compared phases. The modern endcapped octadecylsiloxane-bonded silica phases prepared from high purity silica with a high bonding density have remarkably similar system constant ratios, suggesting near selectivity equivalence, while traditional octadecylsiloxane-bonded silica phases show greater selectivity diversity [258]. Increasing the chain length of alkylsiloxane-bonded phases increases retention through a favorable increase in the cavity and dispersion interaction term and phase ratio that overwhelm the accompanying small changes in the capability for polar interactions. Significant selectivity differences exist between porous polymer and graphitic carbon stationary phases and the octadecylsiloxane-bonded phases, which are complementary rather than equivalent in terms of selectivity. The polar bonded phases are less retentive than the alkylsiloxane-bonded phases due to their smaller m system constants (cavity formation and/or dis-

Table 4.6

System constant ratios for several stationary phases with methanol-water (50:50) as the mobile phase

Stationary phase	System constant ratios				
	<i>m</i>	<i>r/m</i>	<i>s/m</i>	<i>a/m</i>	<i>b/m</i>
<i>(i) Dimethylsiloxane-bonded phases</i>					
Methyl	1.25	0	-0.10	-0.21	-0.79
Cyclohexyl	1.85	0	-0.15	-0.12	-0.76
Octyl	2.29	0.03	-0.26	-0.09	-0.79
Decyl	1.65	0	-0.08	-0.22	-0.76
(CH ₂) ₃ OC ₃ F ₇	1.47	-0.09	0	-0.29	-0.92
(CH ₂) ₂ C ₆ F ₁₃	1.64	-0.17	0	-0.30	-0.85
Phenyl	1.13	0	0	-0.38	-0.78
Pentafluorophenyl	1.56	0	0	-0.22	-0.80
<i>(ii) Octadecylsiloxane-bonded phases</i>					
Hypersil ODS	2.46	0.07	-0.27	-0.08	-0.75
Zorbax ODS	2.68	0.14	-0.31	-0.11	-0.81
Spherisorb ODS-2	2.14	0.17	-0.32	-0.22	-0.86
Capcell Pak C ₁₈	2.23	0.08	-0.21	-0.34	-0.91
J.T. Baker ODS	2.03	0.08	-0.20	-0.17	-0.74
Nucleosil C ₁₈	1.78	0.11	-0.29	-0.25	-0.91
Nucleosil C ₁₈ (HD)	2.37	0.08	-0.16	-0.08	-0.85
Partisil ODS	2.28	0.20	-0.47	-0.21	-0.91
<i>(iii) Other phases</i>					
Porous graphitic carbon (Hypercarb)	3.21	0.30	0.08	-0.07	-0.52
Porous polymer (PLRP-S 100)	2.77	0.16	0	-0.40	-1.01
Horizontally polymerized C ₁₈ /C ₃	2.59	0.17	-0.45	-0.21	-0.91
J. T. Baker Butyl (WP)	1.65	0	-0.15	-0.16	-0.81
J. T. Baker (CH ₂) ₃ CN	0.84	0.25	0	-0.24	-1.05
J. T. Baker (CH ₂) ₃ OCH ₂ CH(OH)CH ₂ (OH)	0.80	0.26	0	-0.20	-1.18

persion interactions with the solvated stationary phase are less favorable for retention). At the same time, they exhibit significant selectivity differences reflected in their system constants for polar interactions. Since some part of these differences result from selective solvation of the stationary phase, the picture realized at a single composition is not necessarily a global picture applicable to all solvent compositions. Some further refinement in our understanding of the stationary phase contribution to retention could be realized if sorbent characteristics such as bonding density, chain length, surface area, etc., were studied in a systematic fashion together with retention modeling using the solvation parameter model. In one case, it was shown that individual system constants for octadecylsiloxane-bonded and butylsiloxane-bonded silica sorbents of similar bonding density were linearly correlated over a wide composition range for mobile phases containing methanol or acetonitrile [270]. Increased retention for the octadecylsiloxane-bonded sorbent was the result of a more favorable contribution from cavity formation and dispersion interactions as well as a more favorable phase ratio with only minor differences due to changes in selectivity for polar interactions.

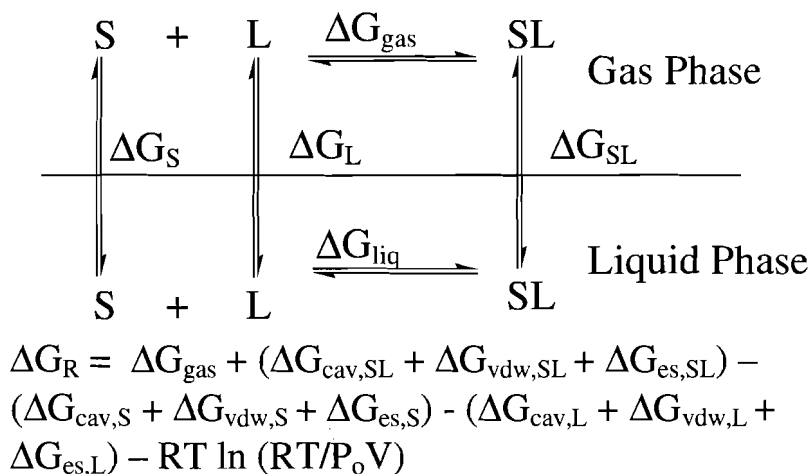
The solvation parameter model has been extended to include ternary solvent systems using a mixture-design approach [271,272]. The system maps are now smooth three-

dimensional system surfaces suitable for the creation of retention maps for method development. In addition, the solvation parameter model has been modified for the conjoint prediction of retention of neutral and ionic compounds by addition of either of two new solute descriptors derived from the acid dissociation constant for the solutes [273-275]. The extended model provided good predictive ability of retention factors for neutral compounds and phenols over the pH range 2-12. In order to combine the structure-driven prediction properties of the solvation parameter model with the commonly used linear solvent strength model, Eq. (4.3), for method development, individual models were constructed for the S -value and $\log k_w$ [261,276-278]. The combined models allow the prediction of retention as a function of binary mobile phase composition but is subject to all the limitations associated with the use of Eq. (4.3). Applications of the solvation parameter model to method development is in its infancy at the time of writing, and a difference of opinion exist concerning the preferred approach [258,278-281]. Notwithstanding these teething problems, early results are encouraging, and it is likely that computer-aided method development approaches for reversed-phase chromatography based on the solvation parameter model will become an important technique.

4.3.1.4 Solvophobic Theory and Lattice Models

The cavity model of solvation provides the basis for a number of additional models used to explain retention in reversed-phase chromatography. The main approaches are represented by solvophobic theory [282-286] and lattice theories based on statistical thermodynamics [287-291]. To a lesser extent classical thermodynamics combining partition and displacement models [292] and the phenomenological model of solvent effects [293] have also been used. Compared with the solvation parameter model all these models are mathematically complex, and often require the input of system variables that are either unknown or difficult to calculate, particularly for polar compounds. For this reason, and because of a failure to provide a simple conceptual picture of the retention process in familiar chromatographic terms, these models have largely remained the province of the physical chemist.

Solvophobic theory explains the retention process in reversed-phase chromatography as a thermodynamic cycle in which binding of the analyte to the stationary phase ligands occurs in the gas phase followed by transfer of the participating species into the mobile phase, Figure 4.14. The free energy change for retention is the difference between the free energy for transferring the ligand-solute complex from the gas phase into solution and the free energy change in transferring the individual components into solution. The transfer free energy for each species is given by the difference in free energy to create a cavity of a suitable size and shape to accommodate the species in the mobile phase and the free energy arising from the interactions between the species and the surrounding solvent molecules. The energy required to prepare a cavity in the mobile phase is equal to the work involved in expanding the liquid surface by the solute surface area. The interaction free energy is composed of essentially two terms: dispersion interactions and electrostatic interactions (dipole, hydrogen bonding, etc.) and an entropic term due to the reduction in free volume accompanying solution of each species from the gas



$$RT \ln K_i = -\Delta G_{\text{gas}} + \Delta G_{\text{vdw,S,i}} + N_A \Delta A_i \gamma_i + N_A A_s (\kappa_i^e - 1) \gamma_i (V_i/V_s)^{2/3} + RT \ln (RT/P_o V_i)$$

Figure 4.14. Diagram of the thermodynamic cycle used to explain retention in reversed-phase chromatography by solvophobic theory. N_A = Avogadro number, ΔA = reduction of hydrophobic surface area due to the adsorption of the analyte onto the bonded ligand, γ = surface tension, κ_i^e = energy correction parameter for the curvature of the cavity, V = molar volume, R = gas constant, T = temperature (K), P_o = atmospheric pressure. $\Delta G_{\text{vdw,S,i}}$ is a complex function of the ionization potential and the Clausius-Moscotti functions of the solute and mobile phase. Subscripts: i = i th component (solute or solvent), S = solute, L = bonded phase ligand, SL = solute-ligand complex, R = transfer of analyte from the mobile to the stationary phase (retention), CAV = cavity formation, VDW = van der Waals interactions, ES = electrostatic interactions.

phase. The mathematical expressions representing these terms are quite complex and in some cases intractable unless it can be assumed that some terms are negligible (see Figure 4.14).

Solvophobic theory successfully predicts:

- The linear relationship between the retention factor ($\log k$) and the volume fraction of organic solvent in binary mobile phases (Eq. (4.3)).
- The increase in retention factor ($\log k$) with increasing chain length for a homologous series (Eq. 4.4).
- The increase in retention factor for neutral solutes in the presence of salts.
- The reduction in retention factors for ionized solutes.
- The linear dependence of the retention factor ($\log k$) on the reciprocal of the absolute temperature (the slope is proportional to the enthalpy of binding).

On the other hand solvophobic theory attributes stationary phase effects to solute-ligand interactions in the gas phase and is unable to account for silanophilic interactions

or analyte interactions with mobile phase components selectively absorbed by the stationary phase. The latter interactions are more likely to be important for polar compounds than the non-polar compounds generally used to validate solvophobic theory.

Lattice theories assume that retention occurs by partitioning of the solute between the mobile phase and the solvated ligands (interphase) region of the stationary phase. A general cavity model is assumed with the driving force for retention by the stationary phase provided by the chemical differences for the contacts of the solute with surrounding neighbors in the stationary and mobile phases and the partial ordering of the bonded ligands, which leads to an entropic expulsion of solute molecules relative to the properties of an isotropic liquid. By changing lattice characteristics the stationary phase can be modeled as an amorphous hydrocarbon oil, liquid crystalline hydrocarbon or an amorphous crystalline hydrocarbon. The liquid crystalline hydrocarbon and amorphous crystalline hydrocarbon models attempt to account for stationary phase organization (bonded chain length, stiffness, surface coverage, etc.) by introducing different degrees of order to the lattice model. The amorphous crystalline hydrocarbon model is perhaps the most appropriate and views the stationary phase ligands as characterized by a gradient of disorder that joins regions of higher order and liquid-like order. The bonded chains are considered to have greater orientational order near the anchored ends than near the free ends. This results in the general expression for retention

$$(1/\phi_B)\ln(k/k_0) = (X_{SB} - X_{SA} - X_{AB}) + \phi_B(X_{AB}) \quad (4.5)$$

where ϕ_B is the volume fraction of organic solvent in a binary mobile phase, k_0 the value of k when $\phi_B = 0$ and X_{SB} , X_{SA} and X_{AB} are the binary interaction parameters among the solute (S), water (A) and organic solvent (B) molecules. The factor k_0 contains the entire stationary phase contribution to retention. For compatible solvents (X_{AB} small) the lattice model predicts a linear dependence of $\log k$ on ϕ_B , Eq. (4.3), and for less compatible solvents a quadratic dependence, Eq. (4.2), as generally observed. A general strength of lattice models is their ability to predict shape selectivity and to accommodate silanophilic interactions.

The partition and displacement model considers retention to result from a two step process. The first involves formation of a mixed stationary phase by intercalation of solvent molecules from the mobile phase. The composition of the solvents in the stationary phase is established according to thermodynamic equilibrium and is usually different to the bulk mobile phase composition. Competitive sorption of solvents is modeled as a displacement process and is complete before the solute is introduced into the two-phase system. Solute retention is then modeled as a partition process between the solvent modified stationary phase and the mobile phase by taking into account all solute-solvent interactions in both phases. The phenomenological model of solvent effects attempts to model retention as a combination of solute-solvent interactions (the solvation effect) and solvent-solvent interactions (the general medium

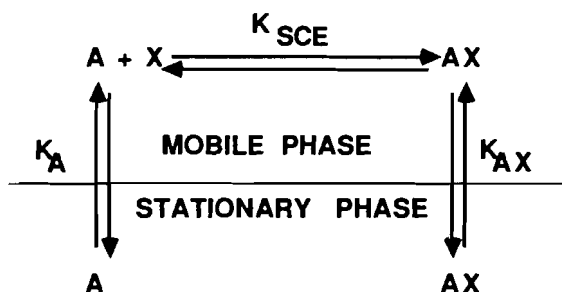


Figure 4.15. General phenomenological retention model for a solute that participates in a secondary chemical equilibrium in reversed-phase chromatography. A = analyte, X = equilibrant, AX = analyte-equilibrant complex, K_{SCE} = secondary chemical equilibrium constant, and K_A and K_{AX} are the primary distribution constants for A and AX, respectively, between the mobile and stationary phases.

effect) for binary mobile phases with the organic solvent as a reference solvent. The solvent effect is modeled as a competitive stepwise solvation exchange equilibrium where water competes with an organic solvent to solvate the analyte. The individual terms representing the different interactions in the partition displacement model and phenomenological solvent effects model are derived by standard thermodynamic methods but are complex and not indicated here.

4.3.1.5 Secondary Chemical Equilibria

In liquid chromatography the primary equilibrium is the distribution of the solute between the mobile phase and stationary phase. By convention all other equilibria in either the mobile or stationary phase are termed secondary chemical equilibria (SCE). Some examples of SCE are ionization (section 4.3.2), ion pairing (section 4.3.3), metal complexation and solute-micelle association (section 4.3.4). Solutes participating in SCE can exist in more than one form. A weak acid, for example, could exist in a neutral or ionized form. The different forms of the solute usually have different retention characteristics in the chromatographic system, but since they are rapidly interconverted, at least on the chromatographic time scale, only a single peak is observed. If this were not the case, either broad and/or multiple peaks would be observed for each solute. The solute retention factor is a function of the weighted-average of the various coexisting solute forms and can be adjusted by changing the parameters that control the solute's equilibrium composition. In this way SCE provides an additional mechanism for control of retention and selectivity. Although some types of SCE can be utilized in the normal-phase mode, the greater ease of application, better understanding of SCE in aqueous or partially aqueous solutions, and the rapid column equilibration times have resulted in the reversed-phase mode becoming the dominant form for practical applications.

The influence of SCE on the primary retention of a solute can be explained in general terms using the simple model depicted in Figure 4.15 [294,295]. The addition of an

equilibrant (X) to the mobile phase introduces a secondary equilibrium that allows the solute to exist in two forms, free analyte (A) and the analyte-equilibrant complex (AX). The concentration of A and AX in the mobile phase also depends on the primary distribution of A and AX with the stationary phase. The equilibrant X is assumed to be unretained, which is a reasonable assumption for acid-base equilibria and some types of ion-pairing, complexation and micellar equilibria, but is not necessarily true in all cases. If the SCE is shifted completely to the left, then the solute will exist solely as species A with a retention factor (k_A) given by the product of the distribution constant (K_A) and the phase ratio of the chromatographic system. If the SCE is shifted completely to the right, then the solute will exist solely as the complexed form AX with a retention factor (k_{AX}) given in a similar manner by the product of the distribution constant (K_{AX}) and the phase ratio of the chromatographic system. The retention factors k_A and k_{AX} are the limiting values for retention of the solute which can elute at any value within the range k_A to k_{AX} depending on the value of K_{SCE} , the equilibrium constant for the SCE. Under intermediate conditions, the retention of solute A will be the weighted-average of k_A and k_{AX} ,

$$k_{obs} = F_A k_A + F_{AX} k_{AX} \quad (4.6)$$

where F_A and F_{AX} are the stoichiometric fraction of the solute in each of its two forms A and AX, and k_{obs} is the retention factor at which the solute is observed to elute under the experimental conditions. F_A and F_{AX} can be expressed explicitly in terms of the equilibrium concentrations of A, X and AX and substituted into Eq. (4.6) to give

$$k_{obs} = K_{SCE} / ([X] + K_{SCE}) k_A + [X] / ([X] + K_{SCE}) k_{AX} \quad (4.7)$$

Equation (4.7) shows that k_{obs} depends on the concentration of the equilibrant [X], the equilibrium constant for the SCE process, K_{SCE} , and the limiting retention factors for the two possible forms of the solute A and AX. The effect of the concentration of equilibrant X on retention depends on the relative values of the limiting retention factors; k_{obs} will increase with increasing [X] if $k_{AX} > k_A$, and vice versa. Since the limiting retention factors are subject to control by other chromatographic variables, such as the identity and concentration of organic solvent, stationary phase properties, ionic strength and temperature, the separation conditions in reversed-phase chromatography using SCE can be quite complex in spite of the simplicity of equation (4.7).

4.3.2 Ion-Suppression Chromatography

Ion-suppression chromatography is used for the separation of weak acids and bases by reversed-phase chromatography [296-299]. The pH of the mobile phase is generally selected to either optimize the band spacing of a series of ionizable substances with different dissociation constants or set to a value to completely inhibit the dissociation of all ionizable compounds. Neutral and ionic substances can be separated simultaneously

but changes in buffer concentration and type, used to change pH, have only a limited, if any effect, on the retention of neutral compounds. The lower efficiency and poor peak shapes observed for some compounds in their ionic form compared with their neutral form or other neutral compounds in the separation tends to favor the use of full ionization suppression. Optimization of separations is then achieved by mobile phase composition variation in the usual way. Otherwise, manipulation of the compounds in their partially ionized form provides greater flexibility for selectivity optimization with the retention factor of ionizable compounds potentially adjustable to any value between that of the fully ionized and neutral forms in any selected mobile phase composition. For strong acids and bases ionization suppression cannot be employed with silica-based chemically bonded phases because of the limited stability of these phases at extreme pH. Either pH stable porous polymer, porous graphitic carbon or zirconia-based polymer coated sorbents, or ion-pair chromatography must be used in this case.

For a monoprotic weak acid [HA], Eq. (4.7) can be written in the familiar form for the dissociation of an acid

$$k_{\text{obs}} = k_{\text{HA}} ([\text{H}^+] / ([\text{H}^+] + K_{\text{a}})) + k_{\text{A}^-} (K_{\text{a}} / ([\text{H}^+] + K_{\text{a}})) \quad (4.8)$$

or

$$k_{\text{obs}} = (k_{\text{HA}} + k_{\text{A}^-} 10^{\text{pH}-\text{pK}_{\text{a}}}) / (1 + 10^{\text{pH}-\text{pK}_{\text{a}}}) \quad (4.9)$$

The equivalent expressions for polyprotic weak acids and bases and zwitterions are given in [287]. From Eq. (4.9) it can be seen that the relationship between k_{obs} and pH is sigmoidal with an inflection point at the pH corresponding to the pK_{a} of the acid in the mobile phase. A small change in the pH near the pK_{a} for the acid results in a large change in k_{obs} but at extreme values with respect to the pK_{a} of the acid ($\text{pH} \geq \text{pK}_{\text{a}} + 2$) changes in k_{obs} are largely dampened.

Eqs. (4.8) and (4.9) are only approximate and require modification if retention is to be modeled in an exact manner [298-301]. The pH and pK_{a} must be expressed with respect to their values for the mobile phase composition and are generally different to their values in water. The proposed models do not consider the effect of the activity coefficient for the ionic species. This can be neglected for water, which has a high dielectric constant, but must be considered for water-organic solvent mixtures. Finally, the hold-up times for the neutral and ionic forms of the solute used to compute the retention factors are generally different because of ion-exclusion effects. In addition the hold-up time for ionic compounds changes with the ionic strength of the buffer. Taking these factors into account a more exact form of Eq. (4.9) can be written as

$$t_{\text{R}} = [(t_{\text{R}}'(\text{HA}) + t_{\text{M}}(\text{HA}))y_{\text{A}^-} 10^{\text{pK}_{\text{a}}-\text{pH}} + (t_{\text{R}}'(\text{A}^-) + t_{\text{M}}(\text{A}^-))] / [y_{\text{A}^-} 10^{\text{pK}_{\text{a}}-\text{pH}} + 1] \quad (4.10)$$

where t_{R} , t_{R}' and t_{M} are the retention time, adjusted retention time and hold-up time of the neutral form of the acid HA or conjugate base A^- and y is the mobile phase activity

coefficient. Eq. (4.10) illustrates that the retention of a weak acid not only depends on the expected solute properties (pK_a , $t_{R'}(HA)$, $t_{R'}(A^-)$) and the pH of the mobile phase but also on the hold-up time and activity coefficient of the anion. The hold-up time and activity coefficient of the anion depend mainly on the ionic strength of the mobile phase. The activity coefficient of the anion can be estimated from the classic Debye-Huckel equation but mobile phase pH and solute pK_a values must be determined by separate experiments.

The inclusion of pH as an optimization parameter presents several practical problems. For expediency the pH of the mobile phase is often taken to be the same as the aqueous buffer before dilution with organic solvent. This is a poor assumption since the pK_a of the buffer, and therefore pH of the mobile phase, depends on the mobile phase composition. Because of the selective solvation of ions by water the pK_a in solvent mixtures containing predominantly water are close to the water value [302,303]. As the proportion of organic solvent increases the pK_a increases in a nonlinear manner eventually approaching the comparatively high values of the pure organic solvent. For cationic acids, such as protonated amines, the opposite pK_a trend is observed. Sometimes the pH is measured after mixing the buffer with the organic solvent. Even in this case, if the potentiometric system is calibrated with aqueous standards the recorded pH is not the correct thermodynamic value for the mobile phase. The correct mobile phase pH is obtained by referencing the measured solution emf to standard buffer solutions of known pH in the same mobile phase composition using the accepted multi-primary standard scale for buffers in methanol-water [301,303-306], acetonitrile-water [299,300,306-309] and tetrahydrofuran-water [306,310,311] mixtures for standardization.

A number of approaches have been described for either pH optimization with a fixed mobile phase composition [242,243,259] or the simultaneous optimization of mobile phase composition and pH [273-275,305,309,312-316]. Typically, retention models are based on the use of Eq. (4.8) or empirical fitting equations with pH or ϕ and volume fraction of organic solvent as variables. Lately attempts have been made to adapt the solvation parameter model or Dimroth and Reichardt polarity parameter (E_T^N) as general system variables to predict retention of ionized compounds [272-274,316]. Method ruggedness often dictates that weak acids and bases are separated in their neutral form at an appropriate pH. The retention factors of partially ionized solutes may vary by as much as an order of magnitude between k_{A^-} and k_{HA} , such that small changes in buffer and mobile phase composition can have a considerable influence on separation robustness. Reversed-phase chromatography can also be used to estimate solute pK_a values in water-organic solvent mixtures by curve fitting the observed retention factor for the solute over a range of pH values with a constant mobile phase composition and ionic strength [297,308,315,317]. A large number of factors affect the quality of the data and it can be debated whether liquid chromatography is the best method for pK_a determinations unless, perhaps, only limited quantities of impure sample are available. Even in this case capillary electrophoresis might be a better choice.

4.3.3 Ion-Pair Chromatography

Ion-pair chromatography is used for the separation of ionic and ionizable compounds and mixtures of neutral and ionic compounds by reversed-phase liquid chromatography [159,318-320]. This technique employs a buffered mobile phase containing an ionic additive (ion-pair reagent) which is responsible for regulating the retention of the ionic compounds but has little effect on the retention of neutral compounds. Ion-pair separations by liquid-liquid chromatography are largely of historic interest today, reflecting the dominance of chemically bonded phases in the contemporary practice of modern liquid chromatography. Separations by liquid-liquid chromatography evolved from extensive research in ion-pair extraction and used similar retention models. Modern theories of ion-pair separations in reversed-phase liquid chromatography do not require or propose the formation of ion pairs. Consequently, numerous names considered more descriptive of the retention mechanism have been proposed for this technique, but with the possible exception of ion-interaction chromatography, widely used in the ion chromatography literature, none of these alternatives have succeeded in gaining popular support.

Ion-pair chromatography is a complementary technique to ion exchange and ion chromatography with all three methods suitable for the separation of similar compounds. The main advantages of ion-pair chromatography are that it does not require special stationary phases or equipment, allows selectivity to be manipulated through changes in the mobile phase composition alone and is suitable for the simultaneous separation of neutral and ionized compounds. Reversed-phase column sorbents provide higher efficiency than fixed-site ion exchangers as well as greater flexibility in method development, since the capacity of the stationary phase for ion interactions is easily varied by changes in mobile phase composition.

There are many variables that affect retention and selectivity in ion-pair chromatography. The most important are the type and concentration of the ion-pair reagent; the pH, ionic strength, buffer composition and concentration and type of organic solvent; stationary phase sorption properties; and temperature. The influence of these variables is often non-linear and interdependent presenting problems for the formulation of simple rules. The most important consideration in selecting a suitable ion-pair reagent is charge compatibility. Surface-adsorbed cations are used to separate anionic solutes and *vice versa*. Quaternary ammonium salts (C_1 - C_4 tetraalkylammonium, C_{10} - C_{18} alkyltrimethylammonium in the halide or phosphate form) are commonly used to separate acids. Protonated tertiary amines (e.g. trioctylamine) are a further possibility for the separation of strong acids, such as sulfonic acids. Alkane- and benzenesulfonates and sulfates (e.g. C_1 - C_{12} alkanesulfonate, camphorsulfonic acid, etc.) are used for the separation of small or polar cations. Large and low-polarity cations are usually separated with small water-soluble counterions, such as dihydrogenphosphate, bromide or perchlorate. For cations of intermediate polarity, hydrophobic counterions, such as naphthalenesulfonate, picrate or bis(2-ethylhexyl)phosphate are used. Solute retention depends primarily on the surface concentration of the adsorbed ion-pair reagent, which is coupled to the composition of the mobile phase, as well as the reagent concentration. Alkanesulfonate ion-pair

reagents of varying chain length result in identical solute retention at similar surface concentrations and increasing retention with chain length at the same mobile phase concentration. At low concentrations retention increases sharply with increasing concentration of ion-pair reagent but eventually reaches a plateau region corresponding to surface saturation by the ion-pair reagent. At still higher concentration retention usually declines due to increasing competition from secondary interactions in the mobile phase that compete with the ion-pair retention mechanism. Ion-pair separations are usually performed in the low concentration or plateau region with typical ion-pair reagent concentrations of 1-10 mM. Since the mobile phase concentration of the ion-pair reagent and organic modifier both affect the surface concentration of the ion-pair reagent, these variables have the greatest effect on the retention of sample ions, and should be used to adjust the mobile phase elution strength. Other considerations in selecting an ion-pair reagent are adequate purity, solubility in useful mobile phase compositions, and minimum interference with low-wavelength UV absorption detection.

The pH of the mobile phase is chosen so that both the sample and ion-pair reagent are completely ionized. Within the normally accessible pH range for alkanesiloxane-bonded silica phases (pH \approx 2-8) this is always the case for strong acids ($\text{pK}_a < 2$) and strong bases ($\text{pK}_a > 8$) but weak acids and bases are only partially ionized. In the latter case retention is expected to increase by optimizing the mobile phase pH to increase the ratio of the ionized to the neutral form of the solute. Important considerations in selecting a buffer to control mobile phase pH are its buffering capacity, solubility in useful mobile phases, ionic strength and suitability for use with low-wavelength UV absorption detection. Inorganic phosphate buffers have remained popular because of their wide pH buffering range but organic buffers, such as citric acid-citrate and alkylamine-phosphate are becoming increasingly popular. Organic buffers are more soluble in water-organic solvent mixtures and provide improved masking of secondary interactions of basic solutes with stationary phase silanol groups, at times responsible for anomalous retention behavior and poor peak shapes. Typical buffer concentrations are 0.5-20 mM, which contribute significantly to the ionic strength of the mobile phase. Increasing ionic strength generally results in an increase in retention. Changes in selectivity and (usually) reduced retention are possible if the buffer ions compete successfully with the analyte for interactions with the adsorbed ion-pair reagent.

The type and concentration of organic solvent can influence retention and selectivity in two ways. Increasing the concentration of organic solvent is expected to decrease retention by increasing the solvent strength of the mobile phase (as generally found in reversed-phase liquid chromatography). Increasing the concentration of organic solvent can also influence retention by (usually) decreasing the amount of ion-pair reagent adsorbed by the stationary phase. Solvent selection, both type and concentration, is limited by the solubility of the ion-pair reagent and buffer in the mobile phase. This tends to favor the selection of methanol and to a lesser extent acetonitrile as solvent strength modifiers. If gradient elution is used, then it is important that the salts added to the mobile phase remain soluble throughout the full range of the gradient composition.

The selection of the stationary phase influences efficiency and the sorption of the ion-pair reagent. Octadecylsiloxane-bonded silica phases are the most widely used with retention increasing generally with the sorptive capacity of the stationary phase. Porous polymers and porous graphitic carbon have been used when the optimum pH for the separation lies outside the suitable range ($\text{pH} \approx 2\text{--}8$) for the alkanesiloxane-bonded silica phases. Increasing temperature tends to reduce retention due to a decrease in the amount of ion-pair reagent adsorbed by the stationary phase. Higher temperatures often provide higher efficiency and lower mobile phase viscosity as well.

Although it would seem that there are many variables to optimize, for simple separations this is not a great problem, since changes in just one or two of the parameters discussed above while maintaining the others within sensible ranges will often yield the desired result. The most important variables are the concentration of the ion-pair reagent, the concentration of organic solvent and pH. Computer-aided strategies are available for simultaneously varying these parameters, and for using defined gradient conditions to estimate reasonable starting conditions for samples of unknown composition [321-325]. For the separation of samples containing both neutral and ionic components the conditions are usually established by selecting a mobile phase composition that provides adequate resolution and separation time for the neutral components. The ion-pairing reagent and buffer are then added to this mobile phase to adjust the separation of the ionic components. Addition of the ion-pairing reagent and buffer usually has little influence on the retention of the neutral components allowing conditions to be selected to move the ionic components to open areas in the chromatogram.

4.3.3.1 *Electrostatic retention models*

Early retention theories were based on stoichiometric models in which either an ion-pair complex was formed in the mobile phase and transferred to the stationary phase or the ion-pair reagent was sorbed by the stationary phase forming a dynamic ion exchanger that retained oppositely charged ions by electrostatic interactions [159,326-332]. Modern electrostatic theories are founded on the principle that long-range coulombic forces give rise to multibody interactions making stoichiometric models fundamentally limited in their ability to explain retention in ion-pair chromatography. Electrostatic theories predict that equilibrium constants prescribed by the stoichiometric models depend on the local electrostatic field strength.

As a consequence of the selective adsorption of ions with a higher affinity for the stationary phase than their counterions electrostatic theories assume the formation of a surface potential between the bulk mobile phase and stationary phase. The adsorbed ions constitute a charged surface, to which is attracted a diffuse double layer of strongly and weakly bound oppositely charged ions equivalent in number to the adsorbed surface charges to maintain electrical neutrality. Because of repulsion effects the adsorbed ions are expected to be spaced evenly over the stationary phase surface and at a concentration that leaves the properties of the stationary phase largely unaltered except for its electrostatic potential. The transfer of solutes from the bulk mobile phase to the

stationary phase is assumed to depend on general solvation and electrostatic effects. General solvation effects depend on the difference in the ease of cavity formation and the strength of intermolecular interactions in the stationary and mobile phases. Neutral solutes are expected to pass unimpeded through the double layer driven by general solvation effects with retention similar to that observed in the absence of the ion-pair reagent [333]. Solutes of opposite charge to the adsorbed ion-pair reagent are driven to the stationary phase by general solvation effects and by their competition with counterions in the diffuse double layer for electrostatic interactions with the charged stationary phase surface. The same factors affect solute ions of similar charge to the adsorbed ion-pair reagent except that now the electrostatic interactions are repulsive and reduce retention.

Several electrostatic theories have been presented which differ in the physical description of the retention process and the role of electrostatic forces [326,329]. Cantwell and co-workers [326,327], for example, have developed a retention theory based on ion-exchange processes in the diffuse double layer combined with surface adsorption, which includes electrostatic interactions. The surface adsorption double layer model developed by Stahlberg and co-workers [328,329] is probably the most explored approach. In this case the surface potential is calculated by assuming a modified Langmuir isotherm for adsorption of the reagent ion and uses Gouy-Chapman theory to derive the relationship between the amount of adsorbed ions and the electrostatic potential of the surface. The retention factor for an ion of charge z at a fixed mobile phase composition is given by

$$\ln k = \ln k_0 + (zF\Delta\Psi_0 / RT) \quad (4.11)$$

where $\Delta\Psi_0$ is the difference in electrostatic potential between the surface of the stationary phase and bulk mobile phase (its sign depends on the charge of the adsorbed ion), k_0 the retention factor for the ion in the absence of the ion-pair reagent in the mobile phase and F the Faraday constant. According to Eq. (4.11), the retention of an ionic solute in ion-pair chromatography is determined by its retention in the absence of the ion-pair reagent, the charge of the analyte ion and the electrostatic surface potential induced by the ion-pair reagent. Eq. (4.11) as presented or in one of its alternative forms, provides a reasonable explanation of the influence of experimental variables on retention for values of $k_0 \geq 0.5$ and for surface concentrations of the ion-pair reagent up to about half the surface monolayer capacity.

4.3.3.2 *Indirect detection and system peaks*

Indirect detection in ion-pair chromatography provides a mechanism for the detection of ions with poor detection properties based on their interactions in the chromatographic system with a immobile-phase ion that possesses favorable detection properties [334-338]. In ion-pair chromatography indirect detection is complicated by the fact that the detection and separation process are coupled and both positive and negative analyte peaks are observed based on the separation mechanism. The detectable ion is added

to the mobile phase at a fixed concentration and equilibrated with the stationary phase before sample introduction, such that the concentration of the detectable ion exiting the column is constant and yields a fixed detector response. Introduction of the sample into the chromatographic system gives rise to a detector response as a result of their influence on the equilibrium of the detectable ion in the chromatographic system. Two types of peaks appear in the chromatogram, which can be either positive or negative in direction with respect to the detector baseline. There should be one peak for each separated analyte in the chromatogram and a number of additional peaks that are characteristic of the chromatographic system (system peaks) that depend on the number of additives in the mobile phase. When the sample ions and detectable ion are of opposite charge the sample ions eluted before the system peak are always negative with respect to the detector baseline and positive if they elute after the system peak. Neutral compounds and sample ions with the same charge as the detectable ion show the opposite response direction with respect to the relative retention of the sample ions (neutral compounds) with respect to the system peak. The direction of the detector response for sample ions, therefore, depends only on their charge type and relative retention with respect to the system peak. The magnitude of the detector response is proportional to the amount of sample ion injected and its retention relative to the system peak. When the analyte and system peak elute close together the response for both peaks is maximized, whereas the charge of the analyte relative to the ion-pair reagent determines whether adequate sensitivity is obtained for the peaks eluting before and after the system peak. Retention of the system peaks is independent of the analyte peaks, although their response direction may change with the composition of the injected sample. Each mobile phase component, except for the main (weak) solvent of the mobile phase can give rise to a system peak if it is involved in the chromatographic equilibrium.

Theoretical models have been developed to explain the appearance of system peaks and to calculate sample detectability in ion-pair chromatography [334,339-342]. When a sample is injected into the chromatographic system, the established equilibrium between the mobile and stationary phases will be perturbed due to the addition of new components (analytes) and deficiencies of other components related to the difference in composition of the mobile phase and sample solvent. The system will strive to reestablish the equilibrium at the point of injection in a rapid relaxation process that adjusts the concentration of all components that are affected by the competition for access to the stationary phase. The sample ion zones migrate along the column in equilibrium with a mobile phase composition that is different to the composition entering the column. The excess or deficit of the detectable ion moving with the sample ion zones is responsible for the direction of the detector response for each sample zone at the detector. The resulting excess or deficit of the mobile phase components outside the sample ion zones move through the column with a characteristic velocity for the distribution of each mobile phase component with the stationary phase and give rise to the system peaks. The system peaks, therefore, are a result of the relaxation process caused by the introduction of the sample in a solvent with a different composition to the mobile phase. The number of observed system peaks depends on

the complexity of the mobile phase and whether each individual component is coupled to the stationary phase equilibria that affects the instantaneous concentration of the monitored mobile phase ion. The concentration of sample ions is generally low and their chromatographic properties are easily predicted assuming normal chromatographic behavior. The sample components resulting in system peaks are generally at relatively high concentration and their retention behavior can only be predicted by nonlinear chromatographic relationships, which are more complex to solve mathematically [342-344]. Co-migration of sample ion and system zones can result in peak deformation and unreliable quantification of the sample ions.

4.3.4 Micellar Liquid Chromatography

Micellar liquid chromatography is a reversed-phase separation technique that uses an aqueous-organic solvent mobile phase containing a surfactant above its critical micelle concentration [345-347]. Retention results from the distribution of solutes between the surfactant coated stationary phase, micelles (pseudophase) and the aqueous-organic solvent portion of the mobile phase. Typical surfactants (sodium dodecyl sulfate, hexadecyltrimethylammonium bromide, polyoxyethylene [23] lauryl ether) have long hydrophobic chains with polar head groups. They are assembled into micelle structures in which the hydrophobic chains form the micelle core and the polar head groups are uniformly distributed in a palisade layer orientated towards the aqueous solution. Micelles are able to solubilize a wide range of organic compounds through surface adsorption, pseudophase extraction (partitioning) and solute-surfactant coassembly (comicellization). Such a range of solute-binding mechanisms is attributed to the heterogeneous structure of micelles and their ability to bind solutes in different microenvironments that depends on the capability of the solute for complementary intermolecular interactions and possibly its concentration. In effect the micelles compete with the stationary phase for solute sorption interactions, and since the micelles are contained in the mobile phase, solute-micelle interactions generally lead to lower retention than observed in the absence of the surfactant. The eluent strength depends mainly on the surfactant and organic solvent concentration. Selectivity is governed by the choice of stationary phase, identity and concentration of surfactant and organic modifier, and for the separation of weak acids and bases, the pH of the mobile phase [348-355].

In general terms, eluent strength in micellar liquid chromatography is inversely proportional to micelle concentration ($1/k \propto [\text{surfactant}]$) and linearly related to the concentration of organic modifier ($\log k \propto [\text{volume fraction of organic solvent}]$). The retention of compounds forming an homologous series is generally nonlinear ($\log k \neq p n_C + q$, see Eq. (4.4)). The retention of ionizable compounds shows a sigmoidal dependence on pH with the retention factor of the acid (HA) and base (A^-) forms of the solute as extreme values, Eq. (4.12)

$$k_{\text{obs}} = (k_{A^-} + k_{HA} K_a^* [H^+]) / (1 + K_a^* [H^+]) \quad (4.12)$$

where K_a^* is the apparent acid dissociation constant, which depends on both the concentration of surfactant and organic solvent, and the association capability of both the acid and base form of the solute with the micelle. Usually K_a^* decreases with increasing concentration of organic solvent and increases with increasing surfactant concentration. It is usually impractical to optimize the surfactant concentration, volume fraction of organic solvent and pH independently because of their interactions. General models used for method development are either entirely empirical containing various combinations of normal variables and their power and/or cross-product terms, or are physicochemical models containing combinations of distribution constants. Fitting either of these model types to experimental data requires an appropriate experimental design to minimize the number of experiments needed to determine the regression or equilibrium constants. Iterative approaches are commonly used with from 4 to 6 experiments required to optimize two variable models for neutral compounds and 12-16 experiments for three variable models for ionizable compounds [350-355]. The pH should be optimized simultaneously with the concentration of surfactant and organic solvent (three variable models), since the change in magnitude of the apparent acid dissociation constants are proportional to the solute-micelle binding constants and can be quite large due to the electrostatic component of the interactions [356]. The MICHROM software provides a complete set of empirical and physicochemical models for optimization of separations in micellar liquid chromatography [345,351].

Water-micelle mobile phases exhibit limited elution strength [357] and poor chromatographic efficiency [358,359] to be generally useful. The exclusion of micelles from the widely used small-pore chemically bonded stationary phases makes a significant contribution to the limited elution strength of micellar mobile phases [360]. Because of the high surface charge on the stationary phase due to surfactant adsorption both electrostatic and steric effects are probably important for the exclusion of ionic micelles. Since the excluded micelles do not have direct access to the solutes except when they diffuse out of the pores, it is not surprising that even high micelle concentrations are not sufficient to elute hydrophobic compounds. Short columns packed with wide-pore and short-chain length, bonded-phase sorbents are used for fast separations or to extend the elution range.

The common organic solvents used in micellar liquid chromatography are n-alcohols (methanol to 1-hexanol), acetonitrile and tetrahydrofuran in amounts usually less than 20 % (v/v) [354,357,361]. The presence of organic solvent reduces the amount of surfactant monomer adsorbed on the stationary phase, changes the elution strength and selectivity of the aqueous portion of the mobile phase and changes micelle parameters such as the critical micelle concentration and aggregation number. If the concentration of organic solvent becomes too high then micelle formation does not occur. The organic solvents affect the system properties in different ways. Methanol, ethanol, 1-propanol, acetonitrile and tetrahydrofuran reside mainly in the aqueous phase. Whereas ethanol and 1-propanol selectively solvate the surfactant head groups promoting micelle formation by reducing the critical micelle concentration, methanol, acetonitrile and tetrahydrofuran have the opposite effect. Because of the smaller size

of these solvents they can more easily solvate the surfactant monomers and hinder micelle formation. Solvents of low water solubility, such as 1-butanol and 1-pentanol, are inserted into the micelle assembly forming swollen mixed-composition micelles and at sufficiently high concentrations they form microemulsions. Because of the different relative distribution of organic solvents between the three compartments of the separation system the influence of solvent type and concentration on selectivity is not as easy to predict as for typical reversed-phase separations. The same general trends are apparent, solute size and electron pair interactions result in increased retention while polar interactions, particularly solute hydrogen-bond basicity, reduce retention (see section 4.3.1.3) [362].

The poor column efficiency observed in micellar liquid chromatography is attributed to slow mass transfer between the micelles and the stationary phase and poor mass transfer within the stationary phase itself [354,358,359]. Slow stationary phase mass transfer can be attributed to poor wetting of the stationary phase with a purely aqueous mobile phase as well as to the adsorption of surfactant monomers, which increases the effective film thickness, viscosity and stiffness of the surfactant-modified bonded-phase. Reasonable efficiency is obtained by operating at lower than normal flow rates closer to the optimum velocity for the system; operating at higher temperatures, e.g. 40°C (at >50°C anionic surfactants significantly increase the rate of silica dissolution); and especially, by adding a small volume (3-5 % v/v) of an organic solvent, such as 1-propanol or 1-pentanol to the mobile phase. The organic solvent probably works by reducing the amount of surfactant adsorbed onto the stationary phase compared with the amount adsorbed in the absence of organic solvent.

The future development of micellar liquid chromatography will probably depend on the development of applications for which micellar mobile phases offer a significant advantage over conventional aqueous-organic solvent mixtures coupled with an increase in efficiency. The direct injection of physiological fluids (surfactants prevent the precipitation of proteins in the chromatographic system) [363], the development of quantitative retention-activity relationships to predict compound hydrophobicity and drug transport [364-367], enhanced detection properties (particularly for fluorescence) [345], fast equilibration in gradient elution, and the low cost of mobile phases are often quoted as strengths of micellar liquid chromatography. The limited number of practical applications would suggest that these characteristics are not sufficient to overcome the limitations of the technique, particularly for analysis. In addition, micellar electrokinetic chromatography, also based on interactions with micellar pseudophases, has been shown to be more adaptable to analytical separations. Although micellar liquid chromatography uses the same equipment and columns as normal reversed-phase chromatography the presence of high surfactant concentrations in the mobile phase increases the requirements for system maintenance [345].

4.3.5 Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) is a reversed-phase separation technique that uses a weakly hydrophobic stationary phase and a negative ionic strength gra-

dient in a buffered aqueous mobile phase for the separation of proteins [5,209-211,368-371]. It provides a complementary approach to ion-exchange and size-exclusion chromatography for the separation and purification of proteins. Low-pressure chromatography using soft gels is widely used for purification and isolation and silica-based chemically bonded and porous polymer microparticle sorbents at higher pressures for analysis. Proteins are usually separated in order of their hydrophobicity with a high probability that their tertiary structure (biological activity) is conserved. Conformational unfolding of proteins on the bonded phase surface as a consequence of the high interfacial tension between the mobile and stationary phases can result in multiple peaks, peak distortions and irreproducible retention for proteins in reversed-phase chromatography. Interfacial tension is minimized in hydrophobic interaction chromatography by using stationary phases of low hydrophobicity together with totally aqueous mobile phases. The elution strength of the mobile phase is controlled by varying the ionic strength rather than by increasing the volume fraction of an organic solvent. Retention and selectivity for the separation of individual proteins depends largely on the stationary phase type, in particular, its bonding density, structure and hydrophobicity. Typical column packings feature a wide-pore substrate with a hydrophilic coating that is lightly substituted with amide, phenyl, short chain alkyl, or alkyl poly(ethylene glycol) groups.

Mobile phase parameters that have to be optimized include the salt type, concentration, gradient shape, pH, temperature, and possibly the addition of a surfactant or organic solvent [368-372]. The change in free energy on protein binding to the stationary phase is determined mainly by the contact surface area between the protein and stationary phase and by the salt type determined by its ability to increase the surface tension of aqueous solutions. Solvophobic theory predicts that in the absence of specific salt-protein interactions and at sufficient ionic strength the logarithm of the retention factor is linearly dependent on the surface tension of the mobile phase, which in turn, is a linear function of the salt concentration Eq. (4.13)

$$\log k = \log k_0 + mM_s \quad (4.13)$$

where k_0 is the retention factor at zero salt concentration, m a parameter reflecting the contact area between the protein and stationary phase, and M_s the molal salt concentration. Non-linearity in plots of $\log k$ against salt molality is due to salt selectivity arising from quantitative differences in salt-specific binding to proteins and/or differential hydration of both stationary phase and protein surfaces. Salts containing a divalent cation and a monovalent anion (e.g. $MgCl_2$, $CaCl_2$) belong to this group. The change in retention with salt concentration varies for different proteins and selectivity depends on both salt type and concentration. An increase in the hydrophobic character of the stationary phase will cause a proportionate increase in the slope, Eq. (4.15), resulting in an increase in retention for all proteins. The magnitude of k_0 depends on the net charge on the protein, which in turn depends on the pH of the mobile phase. Changes in pH affect selectivity due to changes in ionization (increased ionization generally reduces retention) and pH-induced changes

in protein conformation. The strength of hydrophobic interactions generally increases with temperature. Proteins are usually retained longer at higher temperatures, or can be eluted with constant retention by lower ionic strength mobile phases. Not all proteins, however, are conformationally stable at elevated temperatures.

Shallow salt gradients are commonly used to separate protein mixtures. The initial salt concentration primarily affects weakly retained proteins with little effect on well-retained proteins. Higher initial salt concentrations generally result in increased retention in a protein-specific manner. Selectivity is conveniently adjusted by holding the surface tension of the eluent constant while varying the salt type in single-salt gradients, or by varying the salt type and composition in binary and ternary salt gradients. Typical separation conditions employ an ammonium sulfate gradient from 3 to 0 M over 30 min at close to neutral pH (0.1 M phosphate buffer), at ambient temperature, with a 5 to 25 cm column operated at a slightly lower flow rate than used for small molecules. Ammonium sulfate is the most widely used salt combining favorable solubility (≈ 4 M) and strong retention power. Other salts used include ammonium citrate or phosphate and sodium sulfate or chloride. The elution strength is approximately sodium chloride > ammonium phosphate > ammonium sulfate > sodium sulfate > ammonium citrate.

4.3.6 Liquid-Solid Chromatography

Liquid-solid chromatography (LSC) is often referred to as normal phase, straight phase or adsorption chromatography. Separations in liquid-solid chromatography are achieved using a polar stationary phase and a relatively less polar and (usually) non-aqueous mobile phase. Samples are separated based on their competition with the mobile phase for adsorption on fixed or mobility-restrained surface sites on the stationary phase. Appropriate stationary phases include inorganic oxides and polar chemically bonded phases. Porous silica is the most important inorganic oxide phase. Although alumina, zirconia and titania offer complementary separation properties (see section 4.2.1.2), in practice they are of only minor importance. A large number of polar chemically bonded phases have been synthesized for use in liquid-solid chromatography [373], but only siloxane-bonded silica sorbents with 3-cyanopropyl, 3-aminopropyl and spacer bonded propanediol functional groups are widely used for general applications as well as specially prepared chiral stationary phases for the separation of enantiomers.

Liquid-solid chromatography is generally considered suitable for the separation of non-ionic organic compounds soluble in organic solvents. It is indispensable for the separation of samples (analytes and matrices) possessing low solubility in reversed-phase solvents, or when retention and selectivity in reversed-phase liquid chromatography are inadequate. Since retention in liquid-solid chromatography depends mainly on the type and number of functional groups the general order of retention is opposite to reversed-phase liquid chromatography with compounds of low polarity eluting early in the chromatogram and polar compounds towards the end. For silica the general elution order follows the sequence: alkanes < aromatics with one or two rings < halogenated alkanes and aromatics (F < Cl < Br < I) < polycyclic aromatic hydrocarbons < ethers <

nitro compounds < nitriles < most carbonyl-containing compounds < alcohols < phenols < amines < amides < carboxylic acids < sulfonic acids. Internal hydrogen bonding between functional groups and bulky alkyl groups adjacent to polar functional groups diminish retention. The ability of adsorbents to separate solutes into sub-groups based on the type and number of functional groups is the basis of their use in sample preparation procedures for class separations. Also, significant differences in steric fitting of isomers and diastereomers with fixed-position, surface adsorption sites generally make liquid-solid chromatography the best choice for separating these problem compounds.

Retention and selectivity on porous silica stationary phases are dramatically influenced by the presence of even low concentrations of polar contaminants (e.g. water), or compounds intentionally added to the mobile phase (e.g. alcohols, alkylamines, acetonitrile, etc.) called moderators or modulators [11-13]. Beneficial effects include improved retention stability, an increase in sample capacity due to improved linearity of the adsorption isotherms, higher column efficiencies (alcohols are a possible exception), reduced band tailing, and a diminished tendency for sample decomposition. These benefits result from the preferential adsorption of the moderator by the most active sites on the surface of the adsorbent leading to a more energetically homogeneous adsorbent surface. When changing between mobile phases with different hydration levels, column re-equilibration is a slow process (> 20 column volumes of mobile phase may be required to obtain stable retention times). The situation can be even more complex for gradient elution due to changing hydration levels during the course of the gradient and long re-equilibration times to restore the original column activity. Some of these problems can be solved for isocratic separations by using isohydric solvents (solvents with the same hydration level as the adsorbent) [374,375]. For stable chromatographic conditions on silica 50% water saturation of the mobile phase is recommended. Mobile phases with 50% water saturation can be prepared by mixing equal volumes of dry solvent and 100% water-saturated solvent or by using a moisture control system. For gradient elution separations completely dry solvents and constant temperature are recommended [376-379]. Moisture control and its influence on retention reproducibility and system re-equilibration time are problems that can be accommodated but rarely defeated. For routine gradient elution separations reversed-phase liquid chromatography provides more favorable operating characteristics but may not be applicable to all samples separated by liquid-solid chromatography.

Some of the problems discussed for porous silica can be minimized using polar chemically bonded phases. Separations are not as sensitive to variation in solvent hydration levels and gradient elution is facilitated by rapid equilibration with the mobile phase. The surface of the chemically bonded phases is heterogeneous consisting of unreacted silanol groups and the chemically bonded ligands [380-382]. The retention characteristics of 3-cyanopropylsiloxane-bonded silica resemble those of a deactivated (weak) porous silica with unreacted silanol groups acting as the dominant adsorption sites. The addition of small quantities of moderators (< 1 % v/v), such as alkylamines, trifluoroacetic acid, alcohols and perfluorocarbon alcohols, can be used to optimize retention and minimize peak distortions [383]. 3-Cyanopropylsiloxane-bonded phases are

Table 4.7

System constants of the solvation parameter model for liquid-solid chromatography

Stationary phase ¹	Mobile phase	System constants				
		<i>m</i>	<i>r</i>	<i>s</i>	<i>a</i>	<i>b</i>
DIOL	Hexane	-1.05	0	1.63	2.10	3.86
AMINO		-0.85	0	1.40	1.65	3.81
CYANO		-0.37	0	1.88	2.47	0.99
Silica	Hexane-Methanol	-0.83	0	1.06	2.23	1.56
DIOL	(99:1)	-0.85	0	1.07	2.37	1.47
AMINO		-0.72	0	0.94	2.94	1.20
CYANO		-0.61	0	0.95	1.86	1.15
Silica	Hexane-Methyl	0	-0.86	1.67	1.84	3.00
CYANO	<i>t</i> -butyl ether (95:5)	-1.20	0	1.43	1.10	2.80
Silica	Hexane-Methyl	-0.46	-0.21	1.10	1.16	3.02
CYANO	<i>t</i> -butyl ether (80:20)	-1.08	0	1.01	0.53	2.26

¹ AMINO = 3-aminopropylsiloxane-bonded silica; CYANO = 3-cyanopropylsiloxane-bonded silica; and DIOL = spacer bonded propanediol siloxane-bonded silica.

widely used for the separation of basic drugs using liquid-solid and reversed-phase liquid chromatography with an alkylamine moderator or pH buffer to control tailing [384,385]. The retention properties of 3-aminopropylsiloxane-bonded phases are complementary to those of the 3-cyanopropylsiloxane-bonded phase with the amino group imparting strong hydrogen-bonding properties to the stationary phase [380,386,387]. This phase has been used for the class fractionation of polycyclic aromatic hydrocarbons and their alkylated analogs into groups with the same ring number [388]. Amines are easily oxidized and chemically reactive necessitating that some additional precautions are taken when using the 3-aminopropylsiloxane-bonded phase. Mobile phases should be degassed and solvents containing peroxides should be avoided. Samples containing ketones and aldehydes can react with the amino group to form Schiff's base complexes. The spacer bonded propanediol phase (DIOL) is generally described as possessing properties intermediate between those of the 3-aminopropylsiloxane-bonded and 3-cyanopropylsiloxane-bonded phases, exhibiting lower retention for hydrogen-bonding solutes than the former and lower retention of dipolar solutes than the latter [380,382,389].

The above description of the general retention properties of the chemically bonded phases is not entirely satisfactory. The solvation parameter model provides a more quantitative description of system properties [390-393]. Some selected data are summarized in Table 4.7. Retention on all phases results from polar interactions of a dipole-type and through hydrogen bonding. Increasing solute size generally reduces retention and electron lone pair interactions are unimportant for many applications. For the weak solvent hexane the 3-cyanopropylsiloxane-bonded phase is more cohesive and less hydrogen-bond basic than the 3-aminopropylsiloxane-bonded and DIOL phases. The 3-aminopropylsiloxane-bonded and DIOL phases are similar to each other with a slightly different blend of polar interactions. The addition of 1% (v/v) methanol to hexane causes a significant change in selectivity for all bonded phases. The apparent

hydrogen-bond acidity of the stationary phase is strongly reduced and the difference in capability of the stationary and mobile phases for dipole-type interactions is reduced to a lesser extent. The hydrogen-bond basicity of the 3-aminopropylsiloxane-bonded phase is increased significantly and the DIOL phase slightly. With this mobile phase the selectivity of the DIOL phase is very similar to that of silica. With hexane-methyl *t*-butyl ether as mobile phase the apparent stationary phase hydrogen-bond acidity is increased and the hydrogen-bond basicity is significantly reduced. Compared to conditions employed in reversed-phase liquid chromatography the range of selectivity variation is greater for liquid-solid chromatography and more significantly dependent on both the choice and composition of the mobile phase. It is unwise, therefore, to attempt to reduce retention properties to a stationary phase effect alone. A meaningful comparison can only be made for specific stationary and mobile phase combinations, since the same stationary phase can exhibit very different chromatographic properties with different mobile phases. The data in Table 4.7 are also an effective example of the complementary nature of liquid-solid and reversed-phase liquid chromatography. In reversed-phase liquid chromatography solute size is the most important parameter for effective retention with polar interactions tending to reduce retention, the exact opposite of that observed for liquid-solid chromatography. The strong negative effect of solute size in liquid-solid chromatography is probably related to the additional work required to displace an increasing number of solvent molecules from the adsorbent surface to establish the solute in the adsorbed solvent layer. Without compensation by an increase in dispersion interactions for the adsorbed solute compared with the solvated solute this will result in an unfavorable free energy contribution to retention. The data in Table 4.7 also indicates that for solutes with a limited capacity for polar interactions it will be difficult to obtain useful retention in liquid-solid chromatography.

4.3.6.1 Competition Model

The competition model (sometimes referred to as the displacement model) is generally used to describe retention in liquid-solid chromatography. In this model retention results from the displacement of solvent molecules from a surface-adsorbed solvent monolayer with occupation of their position on the adsorbent surface by the solute [207,394-397]. Alternatively, the solvent interaction model relates retention to different adsorption and solute-mobile phase interactions in surface-adsorbed solvent bilayers [9,395,398]. Retention, in this case, can occur by displacement from the primary solvent layer, as for the competition model, or by solute displacement from or association with solvent molecules in the second layer. The competition model, in its expanded form, can explain the main findings of the solvent interaction model as a special case. However, the two models remain distinct in promoting surface adsorption or solvent association as the dominant retention mechanism. In the case of polar chemically bonded phases the competition model provides a reasonable description of retention at low and intermediate concentrations of strong solvent in binary mobile phases. At high concentrations of strong solvent (> 60% v/v) a thicker and more liquid-like stationary phase layer forms, with partitioning becoming the dominant retention process [399,400]. The solvation param-

ter model provides a reasonable explanation of retention for chemically bonded phases but is less successful for silica due to the greater influence of site-specific interactions and inexact modeling of the solute size retention component [390,397]. Spectroscopic studies using attenuated total internal reflection FTIR suggest that retention on silica results from adsorption at two distinct sites identified as isolated silanol groups and surface water adsorbed on vicinal silanol groups [401]. Preferential adsorption at the strong adsorption site initially with further adsorption at the weaker adsorption site at higher surface coverage provided a better explanation of the non-linearity of the adsorption isotherm of ethyl acetate from heptane than either the competition or solvent interaction models. On the other hand 2-propanol exhibited single-isotherm behavior compatible with the competition model, indicating comparable interactions with silanols and surface water due to stronger hydrogen bonding and miscibility with the latter sites. The competition model may not explain all results in liquid-solid chromatography but is the most general and effective approach available.

In its simplest form the competition model assumes the entire adsorbent surface is covered by a monolayer of solute and mobile phase molecules. Under normal chromatographic conditions, the concentration of solute molecules will be small and the adsorbed monolayer will consist mainly of mobile phase molecules. Retention of a solute molecule occurs by displacing a roughly equivalent volume of mobile phase molecules from the monolayer to make the surface accessible to the adsorbed solute molecule. For elution of the solute to occur the above process must be reversible, and can be represented by the equilibrium depicted by Eq. (4.14)



where X represents solute molecules, S solvent molecules, and the subscripts a and m designate molecules in the stationary (adsorbed) and mobile phases, respectively. The competition model thus assumes that a solute molecule replaces n solvent molecules in the adsorbed monolayer, where n is given by the ratio of the adsorption cross section of the solute to that of the solvent. While solute retention can be envisaged as a continuous competitive displacement process between solute and mobile phase molecules on the adsorbent surface, the net retention will depend on the relative interaction energy of the solute and mobile phase molecules with the adsorbent surface. With the simplifying assumptions that the adsorbent surface is energetically homogeneous and that solute-solvent interactions in the mobile phase are effectively canceled by similar interactions in the adsorbed phase, the empirical equations which describe the variation of solute retention as a function of solvent strength, Eq. (4.15) and the variation of solvent strength with composition for a binary mobile phase mixture, Eq. (4.16) were derived.

$$\log k_2 = \log k_1 + \alpha' A_s (\epsilon_1 - \epsilon_2) \quad (4.15)$$

$$\epsilon_{AB} = \epsilon_A + \log [N_B 10^Z + 1 - N_B] / \alpha' n_B \quad (4.16)$$

$$Z = \alpha' n_B (\epsilon_B - \epsilon_A)$$

where k_1 and k_2 are the solute retention factors in mobile phases 1 of solvent strength ϵ_1 and mobile phase 2 of solvent strength ϵ_2 , respectively, α' the adsorbent activity parameter proportional to the adsorbent surface energy, A_s the adsorption cross section of solute X, ϵ_{AB} the solvent strength of the binary mobile phase AB, ϵ_A the solvent strength of the weaker solvent A, ϵ_B the solvent strength of the stronger solvent B, N_B the mole fraction of the stronger solvent B in the binary mobile phase, and n_B the adsorption cross section of solvent molecule B. The adsorbent activity parameter is a measure of the ability of the adsorbent to interact with adjacent molecules of solute or solvent and is constant for a given adsorbent (e.g. 0.57 for silica). The above equations are reasonably accurate for predicting separations involving solute and solvent molecules, which are non-polar, or of intermediate polarity and non-hydrogen bonding. In other cases the model is less successful. For polar solutes and solvents, selective adsorption at high-energy sites is dominant (the adsorbent surface is not energetically homogeneous). As solutes and solvents increase in polarity, their interactions with each other becomes stronger and more specific (solute-solvent and solvent-solvent interactions in the adsorbed and mobile phases do not cancel). These imperfections in the model can be accommodated by either adjusting the parameters in the above equations or by adding additional terms as discussed below.

For weak solvents there is little preference for adsorption on any given part of the surface and little tendency for weakly retained molecules to localize. For more polar solute and/or solvent molecules localized adsorption occurs in which molecules are centered over specific adsorption sites across the surface of the adsorbent and have a higher energy of adsorption than for the non-localized state [380,386,396,402-404]. Experiment shows that localization of the strong solvent B in a binary mobile phase AB will occur provided that the mole fraction of B in the adsorbed monolayer is less than about 0.75. Once localized B molecules cover most of the surface, the remaining spaces on the surface are unable to accommodate B molecules in the configuration required for localization. This factor is referred to as restricted-access delocalization. Restricted-access delocalization is predicted to occur for adsorbents with rigid surfaces and a high concentration of surface adsorption sites, such as silica gel, but is less important for chemically bonded phases due to the relatively low ligand density and the flexibility of the bonded chains. A practical consequence of restricted-access delocalization of polar molecules is that the solvent strength of the mobile phase will vary continuously with the mole fraction of polar solvent in the mobile phase and in the adsorbed monolayer. The separation of two compounds will vary according to Eq. (4.17)

$$\log(k_2 / k_1) = X + Ym \quad (4.17)$$

where X and Y are constants for a given combination of solute and mobile phase composition and m is the mobile phase localization parameter, defined by Eq. (4.18)

$$m = m^\circ f(\theta_B) \quad (4.18)$$

where m° is the value of m for the pure solvent B and $f(\theta_B)$ is some function of the surface coverage of localizing solvent B and non-localizing solvent A. The function $f(\theta_B)$ can have values from 0 to 1 but is not easy to calculate. Details of the calculation method for binary and higher order mobile phases are given in [405,406] and Palamareva has described a computer program suitable for the calculation procedure [407]. The net result of restricted-access delocalization is that the value for θ_B in Eq. (4.18) progressively declines with the surface coverage of the localizing solvent B. The value for θ_B must be adjusted for each mobile phase composition employed when using the competition model.

The retention of polar solutes is also affected by site-competition delocalization. A moderately polar non-localizing solvent molecule can interact laterally with sites upon which a solute molecule is localized. This added competition for the site by both the solute and solvent molecules weakens the net interaction of the solute with the surface. For solvents of increasing polarity a greater decrease in the retention factor with increasing polarity of the non-localizing solvent occurs than is predicted by the simple competition model. This effect can be quantitatively accounted for by assuming a larger value of A_s than is calculated from the molecular dimensions of the solute.

For polar solutes and solvents, particularly those capable of hydrogen bonding, secondary solvent effects due to the specific nature of solute-solvent interactions may also have to be included in the model. The assumption that these interactions are essentially identical in the adsorbed state and mobile phase, and therefore self-canceling, is no longer necessarily true. The addition of a secondary solvent term to Eq. (4.15), in addition to modifying the values for θ_B and A_s due to localization effects, is required to improve the prediction of changes in relative retention of similar solutes [386,405,408].

For strong mobile phase modifiers ($\theta_B \gg \theta_A$) when $N_B \gg 0$ and θ_B does not vary with N_B , Eq. (4.16) can be simplified to the practically useful form

$$\log k = \log k_B - (A_s / n_B) \log N_B \quad (4.19)$$

or

$$\log k = p - q \log (\%B) \quad (4.20)$$

where k_B is the solute retention factor for the pure strong solvent B, p and q are regression constants and $\%B$ is the volume fraction (% v/v) of the strong solvent B in a binary mobile phase mixture [387,395,404]. The slope of a plot of $\log k$ vs. $\log N_B$ is equivalent to the number of solvent molecules displaced by the adsorption of the solute at an active site. When applicable, Eqs. (4.19) and (4.20) are convenient for optimizing chromatographic separations.

4.3.6.2 Solvent Strength and the Eluotropic Series

The calculation of solvent (elution) strength parameters by the competition model is rather involved and a more empirical approach can be justified for routine purposes or for the separation of simple mixtures. For method development an estimate of solvent

Table 4.8

Solvent eluotropic strength for liquid-solid chromatography on silica

Solvent	Type	ϵ°
Hexane, heptane	non-localizing	0.00
Carbon tetrachloride	non-localizing	0.11
Toluene	non-localizing	0.22
Chloroform	non-localizing	0.26
1- or 2-Chloropropane	non-localizing	0.28
Dichloromethane	non-localizing	0.30
2-Propyl ether	minor localizing	0.32
1,2-Dichloroethane	non-localizing	0.34
Diethyl ether	localizing and basic	0.38
Methyl <i>t</i> -butyl ether	localizing and basic	0.48
Ethyl acetate	localizing	0.48
Dioxane	localizing and basic	0.51
Acetonitrile	localizing	0.52
Tetrahydrofuran	localizing and basic	0.53
Acetone	localizing	0.53
1- or 2-Propanol	localizing and basic	0.60
Methanol	localizing and basic	0.70

strength is required to optimize retention and then the selectivity is changed at constant solvent strength by using different solvent modifiers in binary or higher order mixtures [375,409]. The solvent strength of a pure solvent is characterized by the solvent strength parameter, ϵ° , determined by the magnitude of the retention factor for a solute of known cross-sectional area on an adsorbent of known activity for a series of solvents [394,407,410]. Pentane is assigned a value of zero as a reference point. The solvents can then be ordered by increasing magnitude of ϵ° , corresponding to increasing elution strength, known as an eluotropic series, Table 4.8 [375,386,408]. Although ϵ° is an empirical parameter, it can be correlated to general system property scales, such as the solvation parameter model [411], which can be used to estimate ϵ° values when these are unavailable from experiment.

The solvent strength parameter (ϵ°) is defined as the free energy of adsorption of the solvent per unit surface area. Its value depends on both the solvent and the particular adsorbent used [412]. For chemically bonded phases the ϵ° values are substantially smaller and compressed into a narrow range compared with silica. In the simplified competition model differences in solvent strength are assumed to arise exclusively from the differences in free energies of adsorption per unit area. The effects of specific solute-solvent interactions in the mobile phase and on the adsorbent surface are unimportant. However, when the differences in the free energies of adsorption per unit area for different solvents are small, secondary solvent effects become important, and the solvent strength order is then dependent on the nature of the solute. In this case there can be no such thing as a unique eluotropic series applicable to a majority of solutes, and the concept of an eluotropic series loses much of its usefulness. This seems to be the case for carbon and is likely to be the case for the common chemically bonded phases as well.

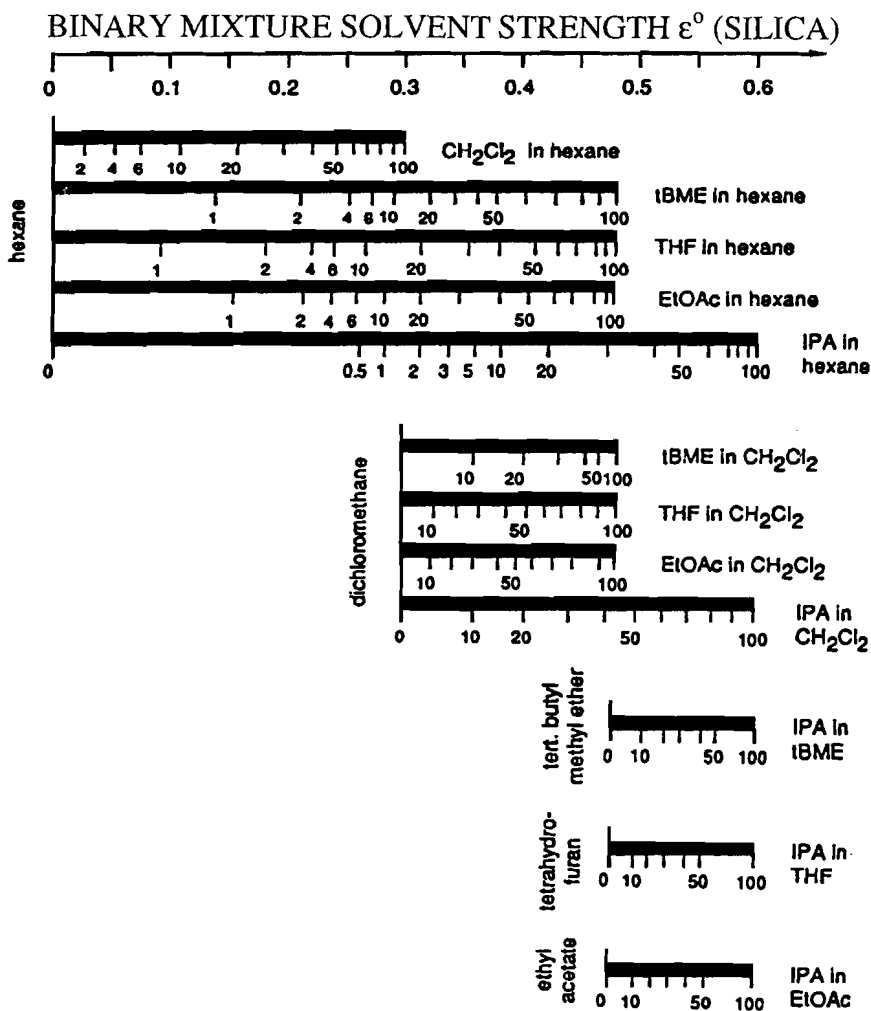


Figure 4.16. Elution strength of solvent mixtures for liquid-solid chromatography on silica gel. Solvent identity: tBME = methyl t-butyl ether; EtOAc = ethyl acetate; IPA = 2-propanol; and THF = tetrahydrofuran. (From ref. [372]. ©Elsevier).

There is no simple relationship between the change in solvent strength as a function of the volume fraction of the more polar solvent. At low concentrations of the polar solvent, small increases in concentration produce large increases in solvent strength; at the other extreme, relatively large changes in concentration of the polar solvent affect the solvent strength of the mobile phase to only a small extent. Graphical methods, such as Figure 4.16, have been developed for obtaining the percent volume composition of binary mobile phases having similar solvent strength but different selectivity [413].

4.3.7 Ion-Exchange Chromatography

Ion-exchange chromatography (IEC) is used for the separation of ions and easily ionized substances (e.g. substances that form ions by pH manipulation or complex formation). One of the principal contributions to retention is the electrostatic attraction between mobile phase ions, both sample and eluent, for immobilized ion centers of opposite charge in the stationary phase. Separations depend on differences in the relative affinity of sample and mobile phase ions for the stationary phase ion centers in a dynamic exchange system, in which sample ions and eluent ions interact with multiple stationary phase ion centers as they pass down the column. For over fifty years ion exchange has been used in clinical and biochemistry laboratories for the routine, automated separation of amino acids and other physiologically important amines to identify metabolic disorders and to sequence the structure of biopolymers [23,209-212,414-417]. Separations are performed on strong cation exchangers with the amino acids in the protonated form using complex pH, buffer composition and sometimes temperature gradients. The amino acids are usually detected after post-column reaction with ninhydrin or o-phthalaldehyde. The separation methods are robust and more than forty physiological amine compounds can be separated in under two hours. These traditional methods are now challenged by new methods using precolumn derivatization with phenyl isothiocyanate, o-phthalaldehyde, etc., and reversed-phase chromatography [417-419]. There is a balance of opinion as to which methods are better, with perhaps the newer methods being preferred for the fast separation of simpler mixtures and the traditional methods for reliable quantification of complex mixtures.

Another traditional application of ion exchange is the isolation, purification and separation of peptides, proteins, nucleotides and other biopolymers on either relatively soft non-denaturing gels for preparative chromatography or wide-pore, microparticle packings for analytical separations [23,369,370,417,420-422]. Reversed-phase chromatography is now the most widely used approach for the separation of peptides but for high molecular mass biopolymers ion exchange offers complementary selectivity and a separation environment that is less denaturing. The latter is often a critical consideration for preparative separations.

Ion exchange has a long history of use in the separation and isolation of carbohydrates [23,417,423-427]. Traditional methods are based on ligand exchange interactions employing cation exchangers loaded with metal ions, such as calcium (recommended for separating sugar alcohols), lead (more effective than calcium for separating similar mono- and disaccharides) and silver (preferred for determining oligomeric distributions). Water or aqueous organic solvent mixtures are used as the mobile phase. Retention is governed by a combination of size exclusion and electrostatic attraction between the electronegative sugar oxygen atoms and electropositive metal cations. Carbohydrates are also separated by anion-exchange chromatography of their negatively charged borate complexes. Since carbohydrates are weak acids ($pK_a \approx 11.9-12.5$) they can be separated by anion-exchange chromatography at high pH (> 12) with sodium hydroxide or sodium hydroxide/sodium acetate solutions as the mobile phase. The carbohydrates are expected to possess a single negative charge per saccharide unit

(regardless of the degree of oligomerization) and elute in order of increasing size and degree of effective ionization. Since few carbohydrates possess useful chromophores for UV-visible absorption detection either pre- or postcolumn reactions to introduce a chromophore or the relatively insensitive refractive index detector were used in early studies. The need for a widely applicable and sensitive detector compatible with gradient elution techniques was solved by the pulsed amperometric detector (PAD), which together with high efficiency pellicular ion exchange packings, is credited with revolutionizing the analysis of complex carbohydrate mixtures.

A major contemporary application of ion-exchange chromatography is the separation of small inorganic and organic ions by ion chromatography [23,159,160,417,428-435]. The accepted use of the term ion chromatography now encompasses a wider range of separation methods than ion exchange, such as capillary electrophoresis and ion-exclusion and chelation chromatography. In which case ion chromatography has become synonymous with a common application and is no longer a description of a single technique for separating ions. This need not concern us here, where we will discuss ion exchange applications only. Ion chromatography evolved from studies in the 1970s that sought to combine the selectivity of ion exchange for the separation of small ions with universal ion detection based on conductivity. In spite of deficiencies in early approaches ion chromatography was quickly accepted in large part because it was able to replace many tedious wet chemical analyses with a simple, automated instrument capable of determining several ions in a single method. In its original form, ion chromatography benefited from the synergy between high efficiency pellicular column packings with a low exchange capacity; the use of low ionic strength mobile phases containing eluent ions with a high affinity for the stationary phase; and the use of a suppressor column combined with flow-through conductivity detection. Modern ion chromatography allows greater flexibility in the choice of detection methods and utilizes a wider range of ion-exchange materials optimized for specific applications. Typical of the ions that can be separated are group I and II cations, NH_4^+ , alkylammonium ions, halide anions, SO_4^{2-} , NO_2^- , NO_3^- , PO_4^{3-} and alkanesulfonates. The current state-of-the-art is indicated by the separation of a complex mixture of inorganic and organic anions in Figure 4.17. In more typical applications a smaller number of relevant ions are separated by well-established isocratic methods. At one time or another most of the metals in the periodic table have been separated by ion exchange using mobile phases containing complexing reagents (alternatively, stationary phases containing immobilized chelate groups can be used, see section 4.3.7.4). Group I and II cations are easily separated using standard ion chromatographic conditions with low capacity cation exchangers and dilute inorganic acid eluents. Polyvalent cations are difficult to separate because they have similar charge to size ratios and similar stationary phase selectivity coefficients. Successful separations require supplementation of the ion exchange mechanism, usually by complex formation in the mobile phase [436-442]. Weak polycarboxylic and hydroxycarboxylic acids (e.g. oxalic, tartaric, lactic, citric, pyridine-2,6-dicarboxylic, nitrilotriacetic and α -hydroxyisobutyric acids) are suitable complexing reagents. These reagents form neutral or charged complexes in the mobile phase. Only the positively

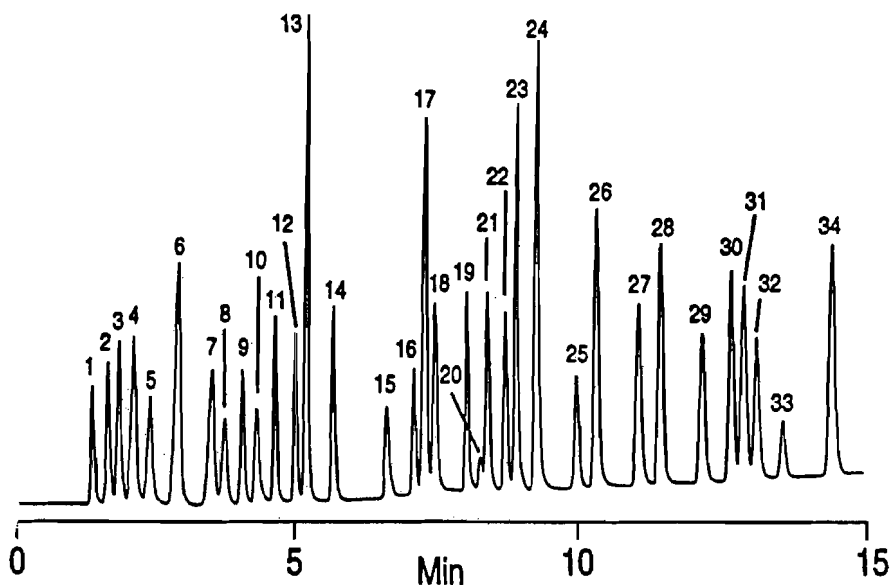


Figure 4.17. Separation of a mixture of inorganic and organic anions by gradient elution ion chromatography with conductivity detection using a micromembrane suppressor. A variable rate gradient from 0.5 mM to about 40 mM sodium hydroxide on an IonPac AS11 column was used for the separation. Peak identification: 1 = isopropylmethylphosphonate; 2 = quinate; 3 = fluoride; 4 = acetate; 5 = propionate; 6 = formate; 7 = methylsulfonate; 8 = pyruvate; 9 = chlorite; 10 = valerate; 11 = monochloroacetate; 12 = bromate; 13 = chloride; 14 = nitrite; 15 = trifluoroacetate; 16 = bromide; 17 = nitrate; 18 = chlorate; 19 = selenite; 20 = carbonate; 21 = malonate; 22 = maleate; 23 = sulfate; 24 = oxalate; 25 = ketomalonate; 26 = tungstate; 27 = phthalate; 28 = phosphate; 29 = chromate; 30 = citrate; 31 = tricarballoylate; 32 = isocitrate; 33 = *cis*-aconitate; and 34 = *trans*-aconitate. Each ion is at a concentration between 1 to 10 mg/l. (From ref. [417]. ©Marcel Dekker).

charged species (free metal cations, cation complexes) or negatively charged complexes take part in the ion-exchange process using a cation or anion exchanger, respectively. Separation selectivity is now controlled by differences in the metal complex stability constants, which are usually more varied than the selectivity coefficients for the metal-ion exchange process. For optimization the most important parameters are the concentration and type of the complexing reagent, mobile phase pH (since most complexing reagents are weak acids) and the type and concentration of the competing mobile phase ions. However, in spite of the success in separating metal cations by ion chromatography the major applications remain the separation of inorganic anions and organic cations in industrial, agricultural, food and environmental samples.

4.3.7.1 Retention Mechanism for Small Ions

General theories for the retention of small ions are based on either stoichiometric or electrostatic double layer models [159,160,329,428,441,443,444]. Stoichiometric models provide a simple picture of the retention process with reasonable predictive

capability. These useful features obscure physical deficiencies in the treatment of electrostatic interactions by the law of mass action. Stoichiometric models fail to consider the effect of long-range, multi-body interactions on the equilibrium distribution constant. Ignoring these problems for the present, stoichiometric models provide a convenient starting point for a general picture of the ion-exchange process. If we assume that the sample and mobile phase ions are in equilibrium with the available ionic sites on the stationary phase, an equilibrium expression can be written in the form of Eq. (4.21)



where A^x represents a sample ion of charge x , E^y a competing mobile phase ion of charge y , and M and S refer to ions in the mobile and stationary phases, respectively. The equilibrium constant is given by

$$K_{A,E} = [A_S^x]^y [E_M^y]^x / [A_M^x]^y [E_S^y]^x \quad (4.22)$$

To be correct the terms in the brackets should be activities and not concentrations, also, two of the terms involve stationary phase activities that cannot be easily evaluated. The weight distribution coefficient for the sample ion A^x is given by $D_A = [A_S^x] / [A_M^x]$ and is related to the chromatographic retention factor for the sample ion by $k_A = D_A w / V_M$, where w is the weight of stationary phase and V_M is the column hold-up volume. Substituting these terms into Eq. (4.22) after rearrangement, we have

$$K_{A,E} = (k_A V_M / w)^y ([E_M^y] / [E_S^y])^x \quad (4.23)$$

As a general condition in ion-exchange chromatography, the sample ion concentration is much lower than the concentration of mobile phase competing ions and $[E_S^y]$ can be replaced by Q / y , where Q is the ion-exchange capacity of the stationary phase. Substituting for $[E_S^y]$ in Eq. (4.23) and rearranging to express the other terms as a function of the retention factor, gives

$$\log k_A = \log (w / V_M) + (1 / y) \log K_{A,E} + (x / y) \log (Q / y) - (x / y) \log [E_M^y] \quad (4.24)$$

For a particular combination of column, mobile phase composition and sample ions $K_{A,E}$, Q , w and V_M will be constant and Eq. (4.24) simplifies to

$$\log k_A = C - (x / y) \log [E_M^y] \quad (4.25)$$

where C is an experimental constant. From Eq. (4.24) retention of the sample ion is determined by the selectivity coefficient, the ion-exchange capacity of the stationary phase, or phase ratio (w / V_M), and the concentration of mobile phase competing ion. Increasing the selectivity coefficient, ion-exchange capacity of the stationary phase or phase ratio increases retention while increasing the concentration of the mobile phase competing ion decreases retention. Increasing the charge on the mobile phase competing

ion (y) decreases retention, whilst increasing the charge on the sample ions (x) increases retention. A plot of $\log k_A$ against $\log [E_M^y]$ should be linear with a negative slope given by the ratio of the charge on the sample and mobile phase competing ions (x / y). A number of separation systems have been shown to obey Eq. (4.25), but exceptions are known, for example, when non-ionic interactions contribute to retention. For electrostatically agglomerated pellicular ion exchangers (see section 4.2.4) deviations from the expected relationship have been attributed to a mixed-retention mechanism involving electrostatic interactions with the oppositely charged core particle [441,445].

The above derivations assume the presence of a single mobile phase competing ion. Mixtures of polyprotic acids and their salts are commonly used as the competing ions in ion chromatography, for example, sodium bicarbonate/sodium carbonate buffers for separating anions. These conditions require modification to the relationships obtained for a single competing mobile phase ion [159,441,444]. The dominant equilibrium approach assumes that the most highly charged mobile phase competing ion is solely responsible for elution and retention can be modeled by assuming that the mobile phase contains this ion only. The effective charge approach assumes that all charged competing ions are responsible for elution of sample ions in proportion to their charges. In Eq. (4.25) the charge y is replaced by the calculated effective charge for the multi-component mobile phase ions. The dual eluent species approach considers both the relative concentrations of the competing ions and their different affinities for the stationary phase. Included in this approach are hydrogen ion concentration, acid dissociation constants and individual ion-exchange selectivity coefficients appropriate for each ion. The agreement with experimental data usually improves with model complexity. The quality of the fit largely reflects the reliability of the assumptions in modeling the real experimental situation.

The equilibrium constant, $K_{A,E}$, described by Eq. (4.23), is called the selectivity coefficient, and is a measure of the preference of the stationary phase for one ion over another [159,160]. Selectivity series have been established for many common ions, Table 4.9. The absolute order depends on the identity of the stationary phase but changes in order are usually minor for common ion exchangers. Polyvalent ions, as a rule, are retained more strongly than monovalent ions owing to their stronger electrostatic interactions with the fixed ionic sites on the stationary phase. For ions of the same charge, retention tends to decrease with increasing hydrated ion radius. The ions in Table 4.9 are listed in approximate order of elution strength and provide a guide to the selection of competing ions for method development.

Electrostatic ion-exchange models attempt to explain retention by the accumulation of ions in the double layer, which is typically a non-stoichiometric mechanism, together with contributions from specific ion interactions with the charged surface as well as changes in ion solvation [329, 443,446]. General models are formulated on the Gouy-Chapman diffuse double layer theory combined with a mass action equation for specific adsorption of both the mobile phase and sample counterions to the charged surface. Ions are treated as point charges and the mobile phase as a continuum with constant polarizability. The stationary phase is regarded as a rigid plane with a fixed number of

Table 4.9

General order of ion-exchange selectivity coefficients

Cations	Anions	
Li ⁺	OH ⁻	Weak competing ions
H ⁺	F ⁻	
Na ⁺	CH ₃ CO ₂ ⁻	
NH ₄ ⁺	Cl ⁻	
K ⁺	SCN ⁻	
Cs ⁺	NO ₂ ⁻	
Ag ⁺	Br ⁻	
Mg ²⁺	I ⁻	
Ca ²⁺	NO ₃ ⁻	
Ba ²⁺	HSO ₄ ⁻	
Cu ²⁺	C ₆ H ₅ O ⁻	Strong competing ions
Ni ²⁺	SO ₄ ²⁻	
	Citrate	

charged groups per unit surface area. Sample ions are present at trace concentrations compared with mobile phase ions. The ion concentration profile is determined by the change in the electrostatic potential as a function of distance from the stationary phase surface and a stoichiometric association constant is used to describe the concentration dependence of specific binding of counterions to the stationary phase surface. The retention factor of a sample ion, k_i , is given by Eq. (4.26)

$$k_i = (A_S / V_M) [\{ \sigma K_{ai} \exp(-z_i F \psi_0 / RT) / -z_i F \} + \int_0^x (-z_i F \psi(x) / RT) - 1) dx] \quad (4.26)$$

where A_S is the stationary phase surface area, σ the surface density of free fixed charges on the stationary phase, K_{ai} the distribution constant for association of the sample ion to the stationary phase surface, z_i the charge on the sample ion, F the Faraday constant, ψ_0 the electrostatic potential of the stationary phase surface relative to the bulk mobile phase, and $\psi(x)$ the electrostatic potential at a point a distance x from the stationary phase surface. From Eq. (4.26) we see that the electrolyte concentration in the mobile phase changes the retention factor of the sample ion through its influence on the double layer, the surface potential, and the concentration of free fixed surface charges on the stationary phase. Numerical solutions for Eq. (4.26) are required because of the integral type and the interdependence between $\psi(x)$, σ and the bulk concentration of mobile phase competing ions. Analytical solutions are possible by imposing restrictions on the variables [443]. Electrostatic models can successfully explain observed experimental deviations from stoichiometric models but are difficult to evaluate.

4.3.7.2 General Considerations for Separating Small Ions

The stationary phase type for a particular separation is selected based on the requirement that the sample ions and immobilized charged groups of the stationary phase must have complementary properties. The separation is then optimized by adjusting the buffer composition (identity of competing ion, ionic strength, pH), temperature, flow

Table 4.10
Experimental factors and their affect on retention in ion-exchange chromatography

Mobile phase parameter	Influence on system properties	Effect on sample retention
Ionic strength.	Solvent strength.	Solvent strength generally increases with an increase in ionic strength. Selectivity is little affected by ionic strength except for analytes with different charges. The type of mobile phase competing ion ($K_{A,E}$ value) controls the strength of stationary phase interactions.
Buffer salt	Solvent strength and Selectivity	Solvent strength and selectivity are influenced by the nature of the counterion i.e., its $K_{A,E}$ value. A change in buffer salt may also change the mobile phase pH.
pH	Solvent strength and Selectivity	Retention decreases in cation-exchange and increases in anion- exchange chromatography with an increase in pH. Small changes in pH can have a large influence on selectivity.
Temperature	Efficiency	Elevated temperatures lead to higher efficiency due to an increase in the rate of solute exchange between the stationary and mobile phases and by reducing the mobile phase viscosity.
	Selectivity	An increase in temperature usually results in a decrease in retention. Small changes in temperature often result in large changes in selectivity, particularly for dissimilar compounds. Since a number of competing equilibria are affected changes can be difficult to predict.
Flow rate	Efficiency	Flow rates may be slightly lower than in other HPLC methods to optimize resolution and mass transfer kinetics.
Organic solvent	Solvent strength	Solvent strength generally increases with the volume percent of organic solvent when hydrophobic interactions contribute to retention.
	Selectivity	The presence of organic solvent modifies mobile phase pH and equilibrium constants that affect selectivity. Changing the type of organic solvent can adjust selectivity as practiced in reversed-phase chromatography for a mixed retention mechanism.
Efficiency		Reduces mobile phase viscosity and improves solute mass transfer kinetics.

rate and type and concentration of organic solvent in the mobile phase, Table 4.10 [159,162,375,447-450]. Increasing the concentration of mobile phase competing ions by either increasing the buffer concentration, or by adding a neutral salt, provides stronger competition between sample and mobile phase ions for the stationary phase ionic centers and generally reduces retention. Since the ionic strength of the mobile phase has the greatest influence on solvent strength, this parameter is usually optimized first to obtain retention factors between 2-20. Selectivity is adjusted by varying the pH of the mobile phase. Changing the mobile phase pH can effect the exchange capacity of weak ion exchangers, the extent of sample ionization, and the effective charge on the competing ions. A pH gradient can be used to optimize solvent strength, but usually an ionic strength gradient is used for this purpose, with narrow range pH gradients used to control selectivity. The operating pH range for a separation can be estimated from the range of sample pK_a values. Since only ionized solutes are retained by an ion-exchange

mechanism, as a general guide, the optimum pH should be 1 or 2 pH units below the pK_a of bases and 1 or 2 pH units above the pK_a of acids. When selecting a buffer salt two criterion must be met. The buffer must be able to establish the operating pH for the separation, and the exchangeable buffer counterion must provide the desired solvent strength. The solvent strength of a buffer can be estimated from the ion-exchange selectivity coefficient for the counterion. Typical buffer concentrations are 1-500 mM.

Mobile phase selection in ion chromatography is restricted by the need to consider detector characteristics as well as other chromatographic properties [159,160,162,428,429, 450]. Many common ions have weak UV-visible absorption properties and a method was needed to detect separated ions in the presence of a highly conducting mobile phase. Since conductivity is a universal property of ions in solution and is simply related to ion concentration, it offers a desirable detection method if the background contribution from mobile phase ions could be eliminated. The introduction of eluent suppressor columns for this purpose led to the general acceptance of ion chromatography. Subsequently suppressor columns have largely been replaced, at first by hollow fiber suppressors, and then by micromembrane suppressors with superior eluent treatment capacity (see section 5.7.4.1). The choice of competing ions for the separation is limited to those ions whose conductivity can be suppressed. For the separation of anions the competing ion must be readily protonated to give a weakly conducting acid. Competing ions that satisfy this requirement include hydroxide, borate, bicarbonate, phenates and some zwitterions, while sodium is a suitable counterion. For the separation of cations the competing ion must be readily hydroxylated to give a weak base. Suitable mobile phases are strong acids (e.g. nitric, sulfuric and methanesulfonic acid), in which the hydronium ion is the competing ion. Water is the weak base formed by neutralization. Changing the competing ion is commonly used to vary selectivity for the separation of anions, whereas the hydronium ion is used almost exclusively for the separation of cations.

It is now realized that the key factor for the detection of ions by conductivity is not that the mobile phase should have low background conductivity, but that the mobile phase ions should have a different equivalent conductance to the sample ions. This opens up many possibilities for direct detection with unsuppressed column systems referred to as single column ion chromatography (SCIC). In SCIC a stationary phase of low capacity (0.01-0.30 meq./g) is used with dilute solutions of electrolytes that have a strong affinity for the stationary phase and a significantly different conductivity to the sample ions for conductivity detection. In most cases 0.1-5 mM solutions of weak aromatic acids, such as phthalic, benzoic and salicylic acids, or their salts, have been used for anion analysis and dilute solutions of strong acids and ethylenediammonium salts for cation analysis. In the SCIC mode, conductivity detection limits are usually poorer and the linear calibration range shorter than for suppressed ion chromatography. For many applications either mode of operation is acceptable.

Further possibilities for detection arise from the fact that the ion-exchange process is an example of a replacement process. Each sample ion peak eluting from the column is really composed of both sample and mobile phase ions. When sample ions elute from the ion-exchange column they replace in the mobile phase an equivalent number of

competing ions to maintain electrical neutrality. It follows, therefore, that if a property of the competing ion is monitored by the detector, then changes in the detector signal will occur upon elution of a sample ion which has a different value of that same property. This is an example of indirect detection, which can be applied successfully to any detection principle, and not just conductivity, provided that there is a large enough difference in the values of the measured property between the competing and sample ions [451,452]. All of the above detection principles are now used widely in modern ion chromatography.

4.3.7.3 Retention Mechanism for Biopolymers

The retention of charged macromolecules in ion-exchange chromatography is a complex process that depends on both the number and distribution of charge sites interacting with the surface of the stationary phase as well as on non-electrostatic interactions [210-212,369,370,421,453-456]. Selectivity can be manipulated through changes in the mobile phase pH and ionic strength, which alter the electrostatic surface potential of proteins. Also, the identity of the mobile phase ions can significantly influence the retention of proteins through their effects on protein solubility and aggregation. Sorptive interactions between proteins and the non-ionic regions of the stationary phase are sometimes important. The high ionic strength of the mobile phase favors hydrophobic interactions by "salting-out" the sample. Size-exclusion effects are a further cause of retention variation by preventing access of the protein to the total pore volume of the stationary phase where the highest concentration of ion exchange sites is located. In addition, since Donnan membrane potentials are developed on charge-carrying supports, ions having the same charge as the stationary phase may be excluded from the pore volume by ion repulsion.

Theoretical models for the ion-exchange separation of proteins are only approximate at best [329,455,457]. The mobile phase competing ions and stationary phase charge centers are usually treated as point charges in electrostatic models but there is no consensus regarding the appropriate geometry for the protein-stationary phase interactions. The protein can be modeled as either a sphere interacting with a planar surface of opposite charge or as a planar surface (slab) interacting with a planar stationary phase. The slab model predicts the generally observed dependence of the retention factor ($\log k$) on the reciprocal of the square root of the mobile phase ionic strength. It also allows the net charge on a protein to be calculated with reasonable accuracy. The slab model also predicts that the slope of the retention plots should be identical for proteins with the same surface area and surface charge density. Although stoichiometric models are less satisfactory than electrostatic theory, they lead to the approximate retention relationship

$$\log k = \log K + z_i \log [1/c] \quad (4.27)$$

where K is a system term (which includes the binding constant for the equilibrium process, the column phase ratio, and the stationary phase ligand concentration), z_i the

number of charges on the protein associated with the sorptive interaction and c is the concentration of the mobile phase competing ion. Exceptions to this equation are common, particularly for separations where hydrophobic interactions are important. At present none of the models are sufficiently sophisticated to explain the ion-exchange retention of proteins over a wide range of experimental conditions.

4.3.7.4 Chelation Ion Chromatography

Weak, chelating carboxylic acids are commonly added to the mobile phase for the separation of polyvalent metal ions by ion-exchange chromatography. Their primary function is to reduce the overall charge on the chelated metal ion. The alternative approach of using stationary phases containing immobilizing chelating ligands for the separation of metal ions is a recent development [436,441,458,459]. High performance chelation ion chromatography (HPCIC), the name usually given to this technique, provides complementary selectivity to cation-exchange chromatography and is relatively insensitive to changes in ionic strength. The low sensitivity of the retention mechanism to ionic strength allows the determination of metals in various complex samples such as selected transition metals and heavy metals in seawater and alkaline earth metals in saturated brines.

Chelation differs from ion exchange in that a labile coordinate bond is formed between the metal and immobilized chelating group. Since common chelating ligands are conjugate bases of weak acids, pH control together with the structure of the chelating group, are the dominant factors that govern the stability of the metal complexes formed in the stationary phase and therefore retention and selectivity. The conditional stability constants of the metal complexes decrease with pH. Relatively small conditional constants appear essential for short retention times and narrow peak shapes. For O,O and O,N chelating ligands (those most commonly used) alkaline earth metals are separated at high mobile phase pH and polyvalent transition and heavy metals at intermediate to low mobile phase pH. Since the range of conditional stability constants for metal chelates is much wider than the range of ion exchange selectivity coefficients, chelation ion chromatography provides greater flexibility over the control of metal selectivity than is possible for cation-exchange chromatography.

Stationary phases for chelation ion chromatography are prepared by two general approaches. The chelating group (e.g. iminodiacetic acid, aminomethylphosphonic acid, or carbamates, etc.) are covalently bound to silica or porous polymer substrates by familiar chemical techniques [458-463]. Selectivity for specific separations is controlled by selection of the immobilized chelate group, variation of mobile phase pH, and sometimes by the addition of a competing chelating reagent to the mobile phase [464]. Mobile phases of low ionic strength allow separations to be optimized by a mixed ion exchange and ion chelation mechanism, providing additional flexibility for the separation of complex mixtures.

In the second approach, the stationary phase is physically coated with a chelating reagent and then conditioned to remove unstable reagent prior to use with a reagent-free mobile phase (precoated stationary phases) [458,465,466]. Alternatively, the

stationary phase is equilibrated by dynamic coating with a chelating reagent present in the mobile phase used for the separation (dynamically coated stationary phases) [465,467-469]. Silica, alkylsiloxane-bonded silica, porous graphitic carbon and porous polymer substrates have been used to prepare physically coated stationary phases. Precoated stationary phases are prepared with triphenylmethane dyes containing the iminodiacetic acid functionality (e.g. xylenol orange, methyl thymol blue, o-cresolphthalein complexone, etc). Low-molecular-mass heterocyclic carboxylic acids (e.g. 2,6-pyridinedicarboxylic acid, 2-quinolinecarboxylic acid, etc.) are used for the preparation of dynamically coated stationary phases. The precoated stationary phases exhibit high metal selectivity but relatively poor column efficiency. Their limited peak capacity restricts their use to the separation of simple mixtures. The dynamically coated stationary phases appear to offer increased sample capacity and improved efficiency. Mobile phases of high ionic strength (e.g. 1 M potassium nitrate) to suppress ion-exchange interactions and containing different amounts of nitric acid to adjust selectivity, are commonly used for separations on coated columns.

4.3.7.5 *Electrostatic Ion Chromatography*

Electrostatic ion chromatography is a new separation technique employing an alkanesiloxane-bonded silica sorbent coated with a hydrophobic zwitterion surfactant [e.g. 3,3-(N,N-dimethyldodeconylammino)-1-propanesulfonate] as a stationary phase and water or a dilute electrolyte solution as mobile phase [470-476]. Inorganic ions are eluted from the column as ion pairs (a pair of ions eluted simultaneously). When pure water is used as the mobile phase, a peak is expected for each possible combination of sample anions and cations. This can lead to complications with a single sample ion appearing as several peaks, each peak containing the sample ion paired with a different oppositely charged ion. Adding an electrolyte to the mobile phase allows the exclusive partitioning and elution of the sample ions as specific ion pairs. The distance between the oppositely charged ion centers of the zwitterion surfactant affects the strength of ion interactions with the coated stationary phase. The elution order of the ions probably results from the balance of interactions between the regionally located cation and anion electrical double layer potentials and the affect of the mobile phase composition on these potentials. This novel form of ion chromatography has yet to yield any significant applications but is mentioned here because of its simplicity and potential for future development.

4.3.8 **Ion-Exclusion Chromatography**

Ion-exclusion chromatography is used for the separation of low molecular weight ions and some neutral substances by a combination of partition, adsorption and ion repulsion [159,428,477-479]. The stationary phase is a high capacity ion exchanger with the same type of immobilized ionic group as the sample ions. Permanent ions with the same charge as the stationary phase are repelled and can only explore the interparticle volume while neutral and partially ionized solutes are retained and separated by partition between the mobile phase trapped in the porous stationary phase and the streaming

mobile phase occupying the interparticle space. In addition, the separation of organic ions often depends on simultaneous contributions from non-electrostatic adsorption and size exclusion by the stationary phase support.

Generally, anions (usually of weak acids) are separated on a strongly acidic cation exchanger in the hydrogen form and are eluted as the neutral or partially ionized acids. Cations (usually protonated bases) are separated on a strong anion exchanger in the hydroxide form and are eluted as the corresponding bases. The mobile phase is often water, although this usually leads to excessive peak fronting for hydrophobic compounds. A better choice in this case are dilute acid solutions (e.g. 1-10 mM H_2SO_4 , HCl , tartaric acid, etc.) or water-organic solvent mixtures. Mobile phases containing weak acids allow unsuppressed conductivity detection to be used [480-482]. For strong acids a membrane suppressor in combination with conductivity detector is required. Direct or indirect UV-absorbance detection is used as a general or selective detector for some ions and neutral compounds [482,483]. General applications include the separation of inorganic ions (e.g. fluoride, phosphates, silicates, nitrite, borate, bicarbonate, etc.), aliphatic and aromatic carboxylic acids, sugars, amines and alkylammonium ions (including ammonium). The separation of low molecular weight carboxylic acids from complex matrices is the most important practical application.

Traditionally, low crosslinked porous polymers modified by sulfonic or carboxylic acid groups (quaternary amines for the separation of cations) were the most widely used stationary phases. In recent years, silica-based chemically bonded or surface-modified (e.g. alumina treated) ion exchangers have found increasing use [159,484-488]. The trend towards increased use of modern porous polymer and silica-based materials is due to their higher performance and greater dimensional stability with different mobile phase compositions.

At low pH weak organic acids are in the neutral or partially ionized form, and can diffuse into the stationary phase pores largely uninhibited by the ionic groups on the stationary phase. Their retention depends primarily on the extent of acid dissociation (they generally elute in order of their pK_a values) and the extent of sorptive (non-polar and polar) interactions of the acid with the stationary phase [488-495]. It is generally found that dibasic acids are less strongly retained than monobasic acids, branched chain aliphatic carboxylic acids are less retained than straight chain acids with the same carbon number, and aromatic (also unsaturated aliphatic) acids are more strongly retained than saturated aliphatic acids. A general retention mechanism to explain this behavior can be written as follows [496,497]:

$$k = [V_S + (K_{\text{ads}}[\text{HR}] + \{K_a / [\text{H}^+]_M\} K_{\text{ads}}[\text{R}^-])V_f] / [1 + (K_a / [\text{H}^+]_M)V_M] \quad (4.28)$$

where V_S , V_M and V_f are the stationary phase pore volume, the interparticle volume and volume of the stationary phase skeleton, respectively; $K_{\text{ads}}[\text{HR}]$ and $K_{\text{ads}}[\text{R}^-]$ the distribution constants for adsorption of the neutral and ionized form of the acid by the stationary phase skeleton, respectively; K_a the acid dissociation constant; and $[\text{H}^+]_M$ the mobile phase hydrogen ion concentration. The retention factor in Eq. (4.28) is valid

for a purely aqueous mobile phase only. In order to accommodate the addition of an organic solvent to the mobile phase the retention factor in Eq. (4.28) is substituted for k_W in the expression $\ln k = \ln k_W + S\phi$ (see section 4.3.1.1), where ϕ is the volume fraction of organic solvent and S is a measure of the solvent strength. Since this expression is only approximate, the same limitations attendant to its general use in reversed-phase chromatography are also applicable here. Eq. (4.28) and its extension indicate the complex relationship between retention and typical operating parameters in ion-exclusion chromatography. The significant contribution to retention from the morphological and chemical properties of the stationary phase skeleton is an indication that substantially different results can be expected for different column materials with the same immobilized ionic groups. In addition to adding an organic solvent to the eluent non-electrostatic contributions to retention can be minimized by incorporating additives [e.g. poly(alcohols), sugars, etc.] in the mobile phase to coat the stationary phase and reduce its sorption characteristics.

4.3.9 Size-Exclusion Chromatography

The origins of modern size-exclusion chromatography (SEC) can be traced to the introduction of crosslinked poly(dextran) and poly(saccharide) gels used for the size separation of water-soluble biopolymers and of semirigid, porous crosslinked poly(styrene) gels for the separation of organic polymers. These developments occurred in parallel with the separation of water-soluble biopolymers being called 'Gel Filtration Chromatography' (GFC), largely by biochemists, and the separation of organic polymers with organic solvents 'Gel Permeation Chromatography' (GPC), largely by polymer chemists. Although both names are still used, this division seems redundant today, and size-exclusion chromatography is the preferred term for all separations resulting from the size-dependent distribution of sample molecules between a mobile phase and a porous stationary phase [23,417,498-505]. The main use of SEC is the separation of macromolecules of defined structure and the determination of the molar mass distribution of polymers with an average structure. Other applications of SEC include sample cleanup (isolation of low molecular mass compounds from complex matrices), desalting of biopolymers and the determination of physicochemical properties (e.g. protein conformation, polymerization kinetics, association and ligand binding constants).

In modern SEC, polymers of an exact molecular weight, such as proteins, and synthetic polymers of a narrow molecular weight distribution, are separated as narrow bands, but with low peak capacity (section 4.3.9.1), and only a few separated bands can be accommodated within the separation. But SEC is still a powerful exploratory method for the characterization of unknown samples, since it provides an overall view of sample composition in a predictable time with little method development (section 4.3.9.2). Polymers of a broad molecular weight range contain many components of similar size that cannot be resolved into individual species, and are eluted as a continuous band with a molecular size distribution that is characteristic of the polymer, for example,

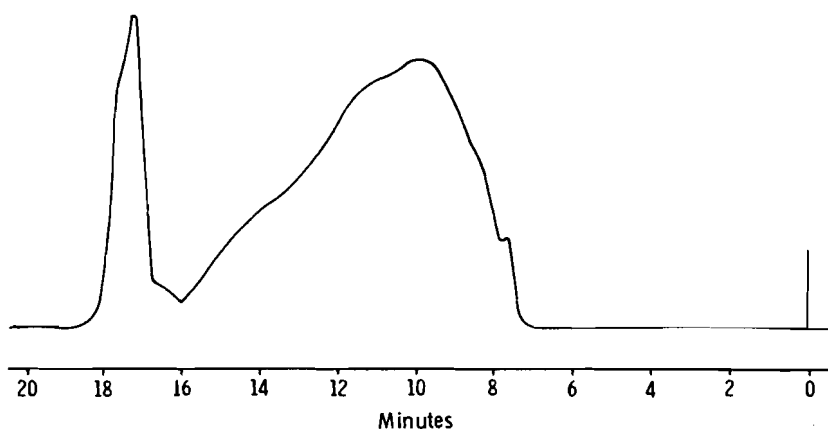


Figure 4.18. Molecular size distribution of a carboxymethyl-cellulose polymer. Column combination of LiChrospher Si-100 and Si-500; mobile phase 0.5 M sodium acetate, pH 6, and flow rate 0.5 ml/min.

Figure 4.18. Often small differences in the chromatographic profile for similar samples can be related to changes in the synthetic procedure or physical and chemical properties of the polymer that affects its performance in an intended application. Size-exclusion separations are not limited to high molecular weight polymers. Gels with mean pore diameters less than 10 nm are available for separating samples with a molecular weight below 500 Da [499,506]. These are useful for the separation of simple mixtures and for the analysis of small molecules in a high molecular weight matrix. In most cases, however, separations using small molecule SEC depend on simultaneous sorption interactions and cannot be rationalized completely within a general size-exclusion mechanism.

4.3.9.1 Theoretical Considerations

Retention differences are controlled by the extent to which sample components can diffuse through the pore structure of the stationary phase, which depends on the ratio of molecular dimensions to the distribution of pore-size diameters. Since no separation will result under conditions where the sample is completely excluded from the pore volume, or can completely permeate the pore volume; the essential requirement for a stationary phase is that the fraction of the pore volume accessible to sample components must be different in a size-dependent manner. Compared with the other liquid chromatographic retention mechanisms discussed so far, a unique feature of SEC is that there are no enthalpic (attractive) stationary phase interactions involved. The driving force for retention is the difference in entropy of the solute within the pore volume compared with that in the moving mobile phase. For SEC the separation is complete when a volume of mobile phase equivalent to the conventional hold-up volume has passed through the column. In other liquid chromatographic techniques, retention is measured as the difference between the elution volume and the hold-up volume, and is always

greater than the hold-up volume. Because of the fundamental difference in elution, the general methods of describing retention and resolution must be modified for SEC [498-501,504,505,507]. In particular, the retention factor and the separation factor have no real meaning in SEC.

Retention in SEC is conveniently described by the distribution function, K_{SEC} , which is related to the experimental parameters by Eq. (4.29)

$$V_E = V_I + K_{\text{SEC}} V_P \quad (4.29)$$

where V_E is the elution (or retention) volume of the solute, V_I the interstitial (or interparticle) column volume and V_P the stationary phase pore volume ($V_M = V_I + V_P$ where V_M is the conventional column hold-up volume). K_{SEC} is constrained to values between 0 and 1, representing the extremes of complete solute exclusion and permeability of the pore volume. It is a function of the size and shape of the molecules being chromatographed with respect to the size and shape of the stationary phase pores. K_{SEC} can be related directly to changes in the entropy of a macromolecule as it penetrates from the mobile phase into a stationary phase pore using appropriate thermodynamic terms and various models for the pore shape and distribution. However, none of these relationships are simple and calibration procedures have been developed to relate K_{SEC} to solute size and indirectly molecular weight and molecular weight distributions (section 4.3.9.3). K_{SEC} also represents the concentration of solute within the pore volume relative to the mobile phase.

The conceptual idea of a theoretical plate can be used in SEC to measure column efficiency as a column quality criterion. For column comparisons it is usually determined with small molecules (e.g. toluene, acetone or benzyl alcohol) which can explore the full pore volume ($K_{\text{SEC}} = 1$). Plate numbers determined in this way produce plate height values lower than those obtained with monodisperse polymers and proteins. With polymers having a defined structure it is legitimate to talk about a plate height value; however, most synthetic polymers are not single substances but mixtures defined by an average molecular size distribution. Under these circumstances the plate height value is an inappropriate measure of column efficiency. The observed peak width is determined by the molecular size dispersion of the polymer as well as by chromatographic dispersion.

The plate height in SEC arises almost entirely from dispersion in the stagnant mobile phase and slow interparticle mass transfer in the streaming mobile phase. Diffusion coefficients for macromolecules are small and the contribution from longitudinal diffusion is insignificant. Resolution in SEC can be improved by minimizing chromatographic peak dispersion by using columns of a small average particle size (3-10 μm) operated close to their optimum mobile phase velocity. Optimum velocities for macromolecules are up to an order of magnitude lower than those used for small molecule separations. Consequently, comparatively long columns packed with small-particle packings can be used at a modest column pressure drop. For broad molecular weight samples, the diffusion coefficients and therefore the plate height and optimum mobile phase velocity, vary throughout the chromatogram.

The specific resolution factor, R_{sp} in SEC, relates peak separations to sample molecular weight, assuming all measurements are made within the linear region of the molecular weight calibration curve, Eq. (4.30)

$$R_{sp} = R_S [1/\log(M_1 / M_2)] \quad (4.30)$$

where R_S is the chromatographic resolution (section 1.6) and (M_1 / M_2) is the molecular weight ratio of two narrow molecular weight standards differing by about 10-fold in average molecular weight. If a chromatographic resolution of unity is accepted as a desirable optimization criterion, then the ratio (M_1 / M_2) can be defined as the minimum molecular weight ratio, R_m . This is a useful parameter for comparing column performance and can be used to relate resolution to the basic properties of the separation system, Eq. (4.31)

$$\log R_m = 4mV_E / V_P \sqrt{N} \quad (4.31)$$

where m is the slope of the linear portion of the molecular weight calibration curve. In order to obtain increased resolving power (corresponding to a minimum R_m value) for a particular column, the chromatographic efficiency and the internal pore volume of the packing should be maximized and the slope of the calibration curve minimized. This can be achieved by using small particles with a narrow particle size distribution, particles with a narrow pore size distribution, particles with a larger pore volume, optimum (low) mobile phase velocities and elevated column temperatures. Due to smaller diffusion coefficients, high mobile phase viscosity and increased sample molecular weight will usually lead to diminished resolution.

The peak capacity (section 1.6.3) in SEC is given approximately by

$$n_{C(SEC)} \approx 1 + [(V_I / V_M) \sqrt{N}] / 4R_S \quad (4.32)$$

Typical values for $n_{C(SEC)}$ are 10-20, considerably smaller than can be obtained with other liquid chromatographic systems, and results primarily from the limited retention range characteristic of SEC.

4.3.9.2 Method Development and Practical Considerations

Method development commences with the selection of the mobile phase. The mobile phase must be a good solvent for the polymer and compatible with the stationary phase and properties of the detector. All separations employ isocratic conditions. The stationary phase must be available in a pore-size range suitable for separating the polymer. Particles of a small size improve resolution, but are less stable at high temperatures and can cause shear degradation of large macromolecules. Particles of 5- μ m diameter are a reasonable compromise for general applications with 10-20 μ m particles chosen for the separation of large macromolecules. Typical column internal diameters are 7.8 mm with smaller diameters used to conserve expensive solvents.

Table 4.11

Suitable mobile phases for polymer separations

Polymer	Mobile phase
Poly(styrene)	Toluene
Poly(butadiene)	Toluene (70°C)
Poly(dimethylsiloxane)	Toluene (70°C)
Poly(ethylene)	Trichlorobenzene (140°C)
Cellulose acetate	Tetrahydrofuran (40°C)
Poly(ester) alkyd	Tetrahydrofuran (40°C)
Poly(methyl methacrylate)	Tetrahydrofuran (40°C)
Poly(propylene glycol)	Tetrahydrofuran (40°C)
Poly(vinyl chloride)	Tetrahydrofuran (40°C)
Poly(urethane)	Dimethylformamide, 0.05 M lithium bromide (85°C)
Poly(imide)	N-Methylpyrrolidone, 0.05 M lithium bromide (100°C)
Poly(acrylamide)	Water, 0.5 M lithium nitrate
Poly(vinyl alcohol)	Water-Acetonitrile (4:1)
Poly(acrylic acid)	Water, 0.1 M sodium nitrate
Poly(ethyleneimine)	Water, 0.5 M sodium acetate, 0.5 M acetic acid

Series coupling of normal-length columns (30 cm) to increase efficiency or to optimize the pore-size range for the separation is common practice. Series coupling of columns is facilitated by the low optimum mobile phase velocity and thus lower column pressure drop per unit column length, typical for SEC separations.

Many polymers can be separated using toluene, trichlorobenzene (140°C), tetrahydrofuran (30-45°C), dimethylformamide (85-145°C), hexafluoroisopropanol and water as a mobile phase, sometimes containing additives to minimize sorptive and ionic interactions with the stationary phase. Some typical examples are summarized in Table 4.11 [21,503,508]. Several common porous polymer stationary phases, although compatible with a number of solvents, swell to different extents in each solvent, and may not allow solvent exchange without deterioration of column properties. Higher temperatures are also quite common in SEC to maintain sample solubility and to minimize mobile phase viscosity.

Either semirigid polymeric gels or rigid, and sometimes surface-modified, silica particles are used for SEC. Poly(styrene-divinylbenzene) and silica or trimethylsilyl-modified silica packings are commonly used with non-aqueous mobile phases. Poly-(methacrylate), poly(propylene oxide) and spacer bonded propanediol modified silica packings are preferred for aqueous mobile phases. A wide variety of specialty packings for sample-specific applications are also available. Column packings are synthesized in a range of single pore sizes between 5 nm and 1 μ m to allow application to a wide range of polymer molecular weights. Various manufacturers describe their single pore-size packings by a single value representing either the average pore size or the size of a standard polymer just excluded from the packing material. When comparing the separation range of different column packings this can cause confusion, since the two estimates of the pore size are unrelated.

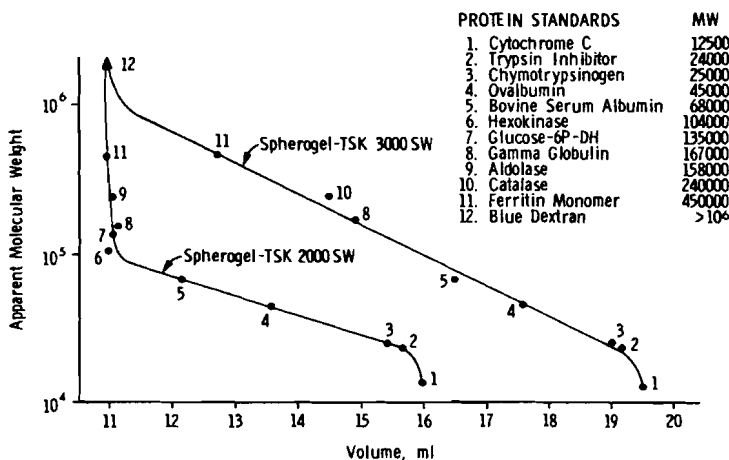


Figure 4.19. Calibration curves for the separation of proteins on Spherogel TSK-SW 2000 and TSK-SW 3000 packings. Mobile phase: phosphate buffer 0.2 M, pH = 6.8.

The shape of the calibration plot for the packing material characterizes the molecular weight separating range, Figure 4.19. The flat central portion of the curve, the fractionation range, represents the useful molecular weight separating range of the packing. The sharp breaks in the calibration plot at either end of the fractionation range correspond to the molecular weight region in which the sample is either totally excluded or free to explore the total pore volume of the packing. In these regions the separation properties of the packing are poor. To separate two components of different molecular size, a stationary phase eluting the two components in the middle of the fractionation range should be selected. For columns of equal efficiency, the packing with the shallowest slope for the fractionation range will provide the highest resolution. Once the optimum stationary phase has been selected, coupling two or more columns of the same type to increase the chromatographic efficiency can be used to improve resolution. Coupling columns that do not contribute to the separation are detrimental to the separation. This is due to the fact that columns that are not relevant for the separation do not contribute to resolution but do contribute to chromatographic dispersion and increase the separation time.

Single pore-size packings are preferred for the separation of polymers with a narrow range of molecular weights. For the separation of polymers with a broad molecular weight distribution either bimodal (or trimodal) mixed-bed columns or series-coupled columns containing different single-pore size packings are required [498,503,509]. Mixed-bed columns containing two or three single pore-size packings with non-overlapping but closely adjacent molecular weight separating ranges (e.g. 6 and 100 nm, or 6, 30 and 300 nm) are generally used. The pore volumes of the packings should be nearly identical to ensure linearity in the calibration plot over the extended molecular weight separating range. Mixed-bed columns provide lower selectivity throughout the

Table 4.12
Maximum sample concentration for polymer separations

Sample molecular weight range	Concentration (% w/v)
0 – 25,000	< 0.25
25,000 – 200,000	< 0.10
200,000 – 2,000,000	< 0.05
> 2,000,000	< 0.02

molecular weight separation range than individual single pore-size packings but provide a wider separating range (about four to six orders of magnitude). The criteria for selecting single pore-size packings for use in series coupled columns are the same as those described for mixed-bed columns. Mixed-bed packings generally provide improved resolution and a wider linear calibration range than is obtained using series coupled columns containing similar packings.

Temperature has little effect on retention in SEC, since strong sorptive interactions with the stationary phase are not involved in the separation mechanism. Temperature differences between the column and mobile phase delivery system are important, however, because temperature gradients cause variations in the elution volume of the polymer due to thermal expansion or contraction of the mobile phase volume. Elution volumes are usually independent of flow rate within the normal flow rate range used in SEC, except perhaps at high column back pressures where mobile phase compressibility or other factors may cause changes in the effective hydrodynamic volume of the polymer. High molecular weight polymers may undergo shear degradation caused by the hydrodynamic forces exerted on the polymer in the sample flow path (column packing and instrument components) [501,502,510]. This has the effect of increasing the concentration of low molecular weight components and broadening the observed molecular weight distribution for the polymer. Macromolecular crowding at high polymer concentrations reduces the hydrodynamic volume of the polymer due to restricted chain motion caused by chain overlap. In addition, macromolecular crowding decreases the conformational entropy of the polymer in the interparticle volume, leading to an increase in K_{SEC} . Recommendations for the selection of sample concentration are summarized in Table 4.12. A difference in sample solution viscosity and mobile phase viscosity exceeding about 10% results in the formation of distorted peaks due to viscous fingering [511,512]. Viscous fingering results from the perturbation of the velocity streamlines as the mobile phase moves through the zone occupied by the sample. When a plug of viscous solution is injected into a mobile phase of lower viscosity, a leading boundary of increasing and a trailing boundary of decreasing viscosity are formed. The leading boundary displaces the mobile phase in front of it without destabilizing the boundary zone. At the rear boundary, however, the low viscosity mobile phase penetrates into the viscous plug, sending fingers of greater mobility ahead of the normal position of the front and leaving behind isolated regions of high viscosity sample solution.

Non-ideal retention behavior in SEC, particularly for poly(electrolytes) in aqueous mobile phases, can render the calculation of molecular weight distributions from chro-

matographic measurements erroneous [500-505,513]. Since polymer-mobile phase interactions play a critical role in controlling the hydrodynamic volume of a macromolecule, mixed mobile phases may lead to deviations from ideal behavior due to preferential solvation of either the stationary phase or macromolecule by one of the mobile phase components. In the case of preferential solvation of the stationary phase, the composition of the mobile phase trapped in the porous support and that in the interparticle volume, are no longer identical. This may cause the hydrodynamic volume of the polymer to change when it enters the packing leading to unexpected elution behavior. Size-dependent changes for poly(electrolytes) depend mainly on the mobile phase ionic strength. At low ionic strengths, electrostatic repulsive forces among neighboring ionic sites expand the polymer chain, increasing its hydrodynamic volume. At higher ionic strengths these electrostatic forces are diminished and the polymer contracts. The extent of contraction depends on the number of ionizable groups and their degree of dissociation, as well as the uniformity and location of these groups on the polymer chain. Also, at low ionic strength, poly(electrolytes) may exhibit expansion at low polymer concentrations. This phenomenon is caused by a decrease in concentration of closely associated counterions surrounding the poly(electrolyte) in proportion to the decrease in poly(electrolyte) concentration. The decreased electrostatic screening among ionic sites on the polymer, leads to an increase in the hydrodynamic volume. A mobile phase of high ionic strength, typically 100-200 mM, is usually required to obtain correct molecular weight information for poly(electrolytes). At this ionic strength the thickness of the double layer becomes negligible.

Stationary phase interactions that affect the retention of poly(electrolytes) include ion exchange, ion exclusion and ion inclusion, as well as various sorptive interactions that depend on non-ionic interactions that apply to all macromolecules. Ionic interactions are caused by the presence of dissociated silanol groups on silica-based packings, carboxylic acid groups on polymer packings, or other ionized groups intentionally or otherwise introduced into the packing material. Optimizing the pH, ionic strength and buffer salts used for the separation minimizes ion-exchange interactions. Also, ionic sites on the support may cause ion exclusion, the process whereby a charged solute is prevented from exploring the pore volume due to electrostatic repulsion by stationary phase ions of like charge. Adding electrolyte to the mobile phase to screen out electrostatic repulsions can be used to overcome this problem. Ion inclusion can result in the appearance of an additional peak in the chromatogram when poly(electrolytes) are separated in a mobile phase containing a salt. In this case, the stationary phase acts as a semipermeable membrane (permeable to small ions but impermeable to macromolecular ions). As the eluent zone containing macromolecular ions travels down the column, the activity of small ions outside of the pores increases because the overall ionic strength increases. Some of the small ions are thus driven into the pores by the difference in activity and are eluted later. Sorptive interactions with the stationary phase are usually controlled by adjusting the properties of the mobile phase so that it competes effectively with macromolecules for interactions with the stationary phase. The most general type

of interactions, hydrophobic interactions, can be eliminated by either adding an organic solvent to the mobile phase or by decreasing the ionic strength of the mobile phase.

4.3.9.3 Molecular Weight Calibration

Separations in SEC are based on differences in size (hydrodynamic volume) which has no direct correlation with molecular weight. The size of a macromolecule in solution is a function of its intrinsic structure and solvent interactions. Some macromolecules are solvated or otherwise associated with small molecules in solution, while others adopt different conformations in response to different solvent environments; such interactions change their hydrodynamic volume and separation characteristics, but not their molecular weight. For monodisperse samples calibration with standards of similar structural properties is required to obtain molecular weight information. For polydisperse samples there is no absolute value for the molecular weight and these materials must be described by some statistical function representing the distribution of different absolute molecular weight species around an average or central value. Suitable terms are the number-average molecular weight ($M_n = \sum N_i M_i / \sum N_i$), the weight-average molecular weight ($M_w = \sum N_i M_i^2 / \sum N_i M_i$) or the z-average molecular weight ($M_z = \sum N_i M_i^3 / \sum N_i M_i^2$) where N_i is the number of molecules having a molecular weight M_i and i is an incrementing index over all molecular weights present. The various molecular weight averages are calculated from the digitized peak profile by manual construction, or preferably by software-based procedures [498,502,514-516]. The high molecular weight portion of the distribution mostly influences the z-average and weight-average molecular weights, whereas the low molecular weight portion has the most influence over the number-average molecular weight. However, there is no preferred choice of molecular weight average for correlating polymer properties to an intended application, and usually the value that shows the most sensible change with the property being correlated is arbitrarily selected. For comparison with absolute molecular weight methods, osmometry yields values for M_n , light scattering M_w , and sedimentation M_z . The polydispersity of a sample is described by the ratio (M_w / M_n) with monodisperse polymers having a polydispersity close to 1.0.

The first step in extracting molecular weight information from a SEC chromatogram is to establish a calibration curve relating the retention volume (or K_{SEC}) to the molecular weight of the polymer [498,502,505,515-517]. Molecular weight, rather than size, is used as the calibration parameter, since the former is independent of the experimental conditions. For monodisperse polymers and macromolecules of absolute molecular weight the primary method of calibration is the peak position calibration method. A series of 4-10 different molecular weight standards are chromatographed under constant experimental conditions and their elution volume measured. For proteins the standards are proteins of known molecular weight in a denatured form to ensure that they all have the same random coil conformation. For synthetic polymers the standards are polymer fractions of narrow polydispersity and identical composition to the sample. A plot of molecular weight against elution volume constitutes the calibration plot as shown in Figure 4.19. The linear portion of the calibration curve is fit to a polynomial

function for conversion of elution volume to molecular weight information. The peak position calibration method is limited in scope by the availability of suitable standards. Although in recent years there has been a significant increase in the number of standards available for commercially important polymers [518,519].

In the absence of suitable primary standards, calibration can be performed using standards of one polymer to calculate the molecular weight of a different polymer. These apparent molecular weight values may be in considerable error in absolute terms and are only suitable for predicting trends. To overcome this problem, a number of attempts have been made to determine a universal calibration parameter characteristic of the effective size of macromolecules [520]. The most widely used parameter is the hydrodynamic volume, which is proportional to the product of the intrinsic viscosity ($[\eta]$) and the molecular weight (M) of the polymer at the same temperature and in the same solvent as the mobile phase [23,498,499,502]. The intrinsic viscosity of the polymer is an experimental quantity derived from the measured viscosity of the polymer in solution or calculated using Mark-Houwink constants ($[\eta] = KM^a$). A plot of $\log([\eta]M)$ against the elution volume provides a calibration curve that is approximately valid for all polymers. To use this approach, a universal calibration curve is first constructed using standards of low polydispersity and the molecular weight of the sample calculated from this curve using the relationship (Eq. 4.33)

$$\ln M_s = [1 / (1 + a_s)] \ln (K_{st} / K_s) + [(1 + a_{st}) / (1 + a_s)] \ln M_{st} \quad (4.33)$$

where the Mark-Houwink constants K and a are known for both the sample, s , and standard, st , for the separation conditions. There are many Mark-Houwink constants reported in the literature, but some are of dubious accuracy, which limits the applicability of this approach.

An alternative method of calibration for when standards of low polydispersity are unavailable is to use a single broad molecular weight standard of the same composition as the sample. The main requirements for this method are: (1) the molecular weight distribution of the standard must span most if not all of the observed distribution range for the sample; (2) the calibration curve must be linear over the entire molecular weight range; and (3) the standard's M_n and M_w values must be accurately known from ancillary measurements. This procedure uses an iterative approach whereby different calibration slopes and intercepts are tried until the correct M_n and M_w values are obtained [521]. The final slope and intercept defines the calibration curve used for molecular weight calculation. A major drawback of this approach is that chromatographic dispersion must be accounted for correctly if accurate results are to be obtained.

The reliability of polymer characterization by SEC has been significantly advanced by new detection strategies based on series-coupled detectors with complementary response characteristics together with appropriate software for calculating molecular weights from the simultaneous detector inputs [518,522-526]. The three most popular detector combinations are refractive index and viscometry, light scattering and refractive

index, and the triple combination of light scattering, refractive index and viscometry. The response of the viscometer detector is proportional to the product of concentration and intrinsic viscosity of the polymer solution and the refractive index detector to concentration. By ratioing detector signals the intrinsic viscosity distribution can be determined leading to the molecular weight distribution by universal or broad molecular weight standard column calibration methods. In addition, the Mark-Houwink constants can be determined from a plot of intrinsic viscosity against molecular weight across the entire molecular weight distribution. Deviation from the expected result provides information about chain branching. The intensity of light scattered by a polymer solution is proportional to concentration and molecular weight and can be determined on-line in SEC using either a low-angle laser-light scattering detectors (LALLS) or multi-angle laser-light scattering detectors (MALLS). There are several different detector designs in production, which measure and treat the light scattering data in different ways. In all cases the combination with a concentration-sensitive detector, usually a refractive index detector, provides a continuous recording of the weight-average molecular weight distribution. The triple detector system eliminates the need for universal column calibration to estimate the molecular weight of polymers of unknown composition. It also allows the calculation of the hydrodynamic volume throughout the entire polymer distribution, which provides important information about the polymer structure and the separation mechanism. In addition, it provides more reliable characterization of polymers containing a small portion of high molecular weight material. Although the use of complementary property detectors together with SEC represents an elegant approach to polymer characterization, the equipment is usually found only in laboratories specializing in polymer analysis. Band broadening in interdetector volumes is a cause for concern in the characterization of narrow molecular weight polymers [526,527].

4.3.10 Gradient Polymer Elution Chromatography

Molecular weight distribution information obtained by size-exclusion chromatography on its own is insufficient to characterize the properties of complex polymers, such as copolymers and block and graft polymers [23,514,524]. For these polymers the chemical composition and functionality type distributions are equally important. A major obstacle to the characterization of these materials is that their molecular properties are present as joint distributions. Unlike the mass distribution the composition and functionality distributions can only be determined by separation methods that employ interactions with the stationary phase. To fully characterize a complex polymer it is not unusual to use manual or automated tandem techniques where the sample is fractionated according to its chemical or end group composition for subsequent further separation by size-exclusion chromatography to establish their mass distribution. Chromatographic methods may also be combined with spectroscopic methods to determine microstructural information.

The methods used to obtain chemical composition and functionality distributions for complex polymers are based on precipitation/redissolution, sorption and exclusion ef-

fects in solvent gradients [528-530]. These techniques are known as precipitation chromatography, sudden-transition gradient chromatography, liquid-adsorption chromatography and liquid chromatography under critical conditions (of adsorption). Typical separations may rely upon a multiplicity of retention mechanisms and the term gradient polymer elution chromatography (GPEC) can be used as a generic expression for all techniques without defining an individual separation mechanism.

The number and range of solvents suitable for the dissolution of a polymer is more restricted than for low molecular mass solutes of similar structure. The solubility of a polymer in a mixed solvent containing increasing amounts of a non-solvent is inversely proportional to the square root of the polymer molecular weight. This dependence is the basis of the separation of polymers by either fractional precipitation or dissolution. Temperature is also an important parameter for controlling precipitation and dissolution. For separation, at least part of the polymer sample is precipitated at the head of the column by injection into a mobile phase that is a poor solvent for the polymer. Individual polymeric species are subsequently eluted in a solvent gradient of a good solvent added continuously in increasing amounts to the non-solvent. The separation order depends on the relative polarity of the non-solvent to the good solvent (the non-solvent can be more polar than the good solvent or *vice versa*). Each polymeric species is dissolved during gradient elution at a mobile phase composition characteristic of its molecular weight and chemical composition [530-535]. In the case where the elution strength of the mobile phase is sufficient to exclude adsorption on the stationary phase, the polymers are eluted in a solvent mixture corresponding to the cloud point in almost quantitative agreement with observations on polymer solubility made by turbidimetric titrations. In this case the separation does not depend on the identity of the stationary phase. When the dissolved polymers are additionally adsorbed on the stationary phase they are eluted at a higher concentration of the good solvent than predicted from polymer solubility data. The application of chromatography under sorption conditions is especially useful for the determination of the chemical composition distribution of copolymers, the separation of polymer blends and the fingerprinting of resins. With porous stationary phases the velocity of the excluded polymer fractions is faster than that of the eluent front (solvent is not excluded from the pore volume) and the polymers are eluted as colloidal solutions which can cause problems with photometric detection resulting from simultaneous absorption and light scattering. This results in an increased photometric response with increasing polymer molecular weight. These conditions should be avoided for quantitative analysis. In sorption systems it is necessary to adjust the separation conditions to minimize the influence of sample load on retention. Higher sample concentrations cause increased retention in solubility-controlled separations but decreased retention in adsorption-controlled systems. Adjusting the mobile phase composition or temperature can counteract the influence of sample load on retention [531].

Sudden-transition gradient polymer elution chromatography is used to obtain separate control of the sorption and precipitation/dissolution retention mechanisms [530,536,537]. In a typical binary gradient the solvent power and polarity of the mo-

bile phase are changed simultaneously throughout the separation. More promising for polymer separations are ternary systems consisting of two non-solvents (A and B) and a good solvent (C). The two non-solvents must be of opposite polarity and the good solvent of intermediate polarity (between A and B) and miscible with both non-solvents. The polymer dissolved in the good solvent is injected into the separation system containing one of the non-solvents and precipitated at the head of the column. The concentration of the good solvent is abruptly changed to a concentration sufficient to dissolve the polymer without elution. Polymer species are then eluted by a polarity gradient generated from the two non-solvents at a constant concentration of the good solvent.

Critical conditions for the separation of polymers are defined as those conditions where entropic exclusion effects are exactly compensated by enthalpic adsorption effects [524,530,538-541]. Under these conditions, retention is solely governed by small differences in the chemical structure of the polymer chains. At the critical point of adsorption all members of a homogeneous polymer elute in a narrow peak at the same elution volume regardless of chain length. In this case retention is determined by the energetic interactions of the functional groups of polymer species with the stationary phase. For polymers with a heterogeneous structure individual species with identical functional groups elute at different elution volumes characteristic of the composition heterogeneity of the polymer. This allows the determination of the functionality type distribution of a polymer. For block copolymers, separation under critical conditions of adsorption for one block allows a separation according to the length of the other block, which can be utilized for the determination of the chemical composition distribution. The same approach can be applied to graft copolymers, which can be separated under critical conditions of adsorption for the grafts according to the length of the backbone or *vice versa*. Suitable conditions for separations under critical conditions of adsorption are searched for by varying the ratio of good solvent to non-solvent at different temperatures. There is no simple process to establish optimum separation conditions and several practical problems leading to peak splitting or broadening require experimental control.

MALDI (matrix assisted laser desorption and ionization) combined with size-exclusion chromatography is increasingly being used to study the microstructure of polymers [542-544]. MALDI allows the desorption and ionization of macromolecules with molecular mass up to hundreds of kilodaltons with little or no fragmentation. The MALDI mass spectra provides structural information for identification of the polymer repeat units or copolymer sequence, end group mass and mechanism of polymerization for polymers with a narrow polydispersity (< 1.25). For samples of high polydispersity MALDI fails to provide reliable information. The separation of polydisperse samples by size-exclusion chromatography provides fractions suitable for characterization by MALDI. MALDI can also be used for mass calibration of size-exclusion columns.

4.3.11 Liquid-Liquid Chromatography

Interest in liquid-liquid chromatography (LLC) has declined dramatically over the last 10-15 years as chemically bonded phases became widely accepted for separations and

centrifugal countercurrent chromatography for solute property determinations [545-547]. The main problem in LLC of stationary phase erosion can be largely overcome by solvent-generated LLC, where the stationary liquid phase is generated dynamically by the mobile phase [547-549]. In this approach, one of the phases of an equilibrated liquid-liquid system is applied as a mobile phase to a solid support, which is preferentially wetted by the other phase of the liquid-liquid system. The support is usually silica when the stationary phase is aqueous or a polar solvent and a chemically bonded support when the stationary phase is a non-polar solvent. Although sufficiently strong adsorptive properties are required to obtain wetting, some compromise is required, since the support should have negligible adsorptive properties for the sample. In most cases the pores of the support will be completely filled with the stationary liquid phase. To adjust the phase ratio of the column, a support with a different surface area and specific pore volume is selected. To minimize stationary phase erosion the sample solvent should have a similar composition to the mobile phase and gradient elution is generally not possible.

Solvent-generated stationary phases allow more stable and reproducible systems to be prepared compared with conventional preparation techniques. The latter rely on loading the column from a solvent in which the stationary phase is soluble followed by displacing the solvent and excess stationary phase from the column with mobile phase saturated with stationary phase. The slight mutual solubility of the two phases makes these systems unstable. Long-time operation usually requires presaturation of the mobile phase with stationary phase and thermostating of the mobile phase and column to avoid fluctuations in the phase ratio and displacement of the stationary phase from the column.

In the absence of interfacial adsorption, retention in LLC is simply defined by Eq. (4.34)

$$V_R = V_M + K_D V_S \quad (4.34)$$

where V_R is the retention volume, V_M the volume of mobile phase, V_S the volume of stationary liquid phase, and K_D the liquid-liquid distribution constant. V_M and V_S are usually calculated from the intercept and slope, respectively, of the plot of V_R against K_D , where values of K_D for a number of test solutes are determined in a static liquid-liquid distribution experiment. Once values of V_M and V_S have been determined, values of K_D for different solutes are simply obtained from the elution volumes of the solutes in the same chromatographic system. Deviations from linearity in the plot of V_R against K_D or tailing of polar compounds are symptomatic of interfacial adsorption.

4.4 METHOD DEVELOPMENT

Method development in liquid chromatography is often time consuming for all but the simplest of separations, and can be a frustrating experience for the less experienced



Figure 4.20. Outline of the steps involved in developing a method for a separation by liquid chromatography.

chromatographer [375,550-552]. This is due primarily to the diversity of retention mechanisms available, the large number of interdependent parameters associated with each method and the fact that several chromatographic separations and associated decision steps are usually required to arrive at suitable separation conditions. Computer simulation methods can be helpful, but even these require a certain amount of experimental data and call for decisions from the operator before predictions can be made. The expert analyst relies upon a lifetime of experiences to answer the many questions that arise in the decision making steps to develop a suitable method, but does so in a manner that is not simply systematized for the benefit of the less experienced analyst. The best that can be hoped for in this section, therefore, is to provide some logical guidelines as to how a method is generally developed, without belief that a panacea for all problems is possible. At least by eliminating those decisions or experiments from the method development process that are least likely to yield useful information, the process should be both faster and more logical.

The general steps in developing an acceptable analytical method in liquid chromatography are summarized in Figure 4.20. Method development starts with a clear statement of the needs of the analysis. How many detectable compounds are present in the sample? Are all peaks equally relevant? In the first case all peaks must be adequately separated and the difficulty of providing the desired result will increase with the number of sample components. In the second case the problem is truncated to the separation of the relevant peaks from each other and from the sample matrix (irrelevant peaks) as a group. Any method development strategy will be simplified if the number of relevant sample components is known in advance. This is the only way to be certain that the separation is complete. The availability of standards for the compounds of interest assists in tracking their position during optimization of the separation. The concentration range of relevant peaks and the detection characteristics of the sample components dictate the choice of detector, which in turn restricts the choice of mobile phase solvents to those that are compatible with the detector. When only a few components are of interest the properties of the remainder, now considered the matrix, can have a bearing on the compatibility of the sample with the separation system and whether additional sample preparation steps will be required before the separation step. Also, the separation time may be an implied constraint for the separation. All the above factors are important in reaching decisions that affect each subsequent step in the method development process.

To select a separation mode, the analyst usually relies on personal insights and experiences gained with similar samples in the past, information taken from the literature describing the separation of the same or similar samples, and/or the availability of certain columns and equipment. The last approach is based on expediency rather than scientific rigor and is not considered here. Some general information,

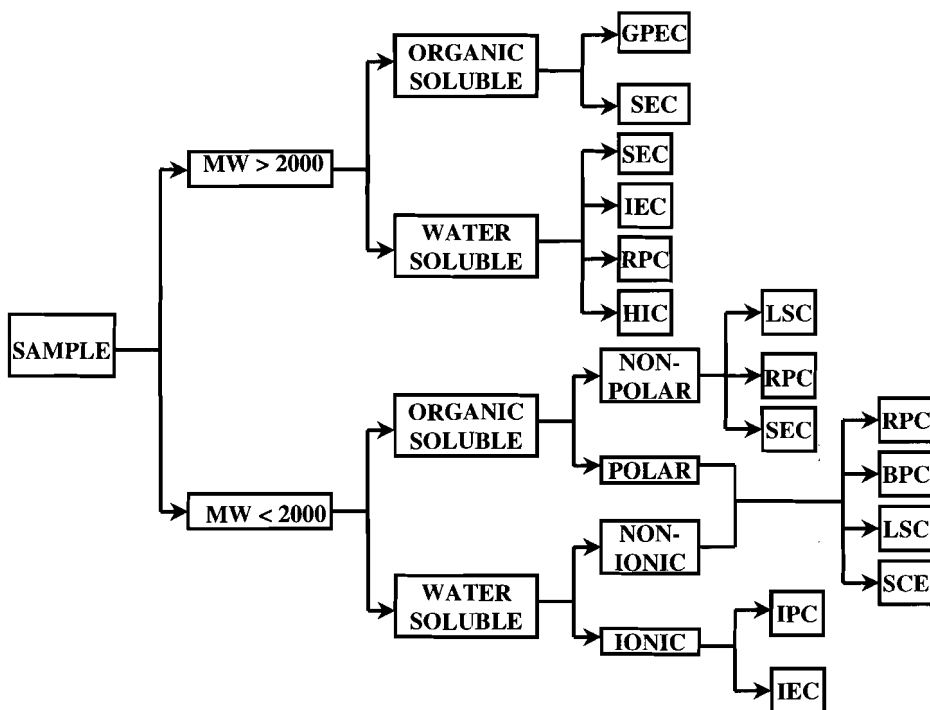


Figure 4.21. Column selection guide based on sample type (MW = molecular weight). GPEC = gradient polymer elution chromatography; SEC = size-exclusion chromatography; IEC = ion-exchange chromatography; HIC = hydrophobic interaction chromatography; LSC = liquid-solid chromatography; RPC = reversed-phase chromatography; BPC = (polar) bonded-phase chromatography; and IPC = ion-pair chromatography.

accessible through simple experiments when not already known, such as solubility in different solvents, component molecular weights, acid dissociation constants and conductivity can be used to select a suitable retention mechanism, as indicated in Figure 4.21. The molecular weight cut off at 2000 is quite arbitrary and reflects the fact that size-exclusion packings are readily available for the separation of higher molecular weight compounds. Packings for small molecule size-exclusion separations are available but have a limited peak capacity restricting their use to the separation of simple mixtures. Likewise, wide pore packings permit the separation of polymers with a molecular weight exceeding 2000 by reversed-phase and ion-exchange chromatography, for example.

Some separation techniques have greater scope than others do for the separation of certain sample types. For example, if the information sought is the molecular weight distribution of a sample, then size-exclusion chromatography (section 4.3.9) is an obvious choice. Liquid-solid chromatography (section 4.3.6) is often preferred for the separation of structural isomers of moderate polarity. The separation of members

of a homologous or oligomer series is generally best accomplished using reversed-phase chromatography (section 4.3.1). Weak acids and bases are usually separated by ion-suppression reversed-phase chromatography (section 4.3.2) or by ion-exchange or ion-exclusion chromatography. Strong acids and bases are usually separated by either ion-pair (section 4.3.3) or ion-exchange chromatography (section 4.3.7). Enantiomers require special stationary phases containing suitable chiral centers for their separation and are discussed elsewhere (section 10.4). In some cases several approaches will lead to an acceptable separation and method development is not particularly difficult. In other cases the selection of appropriate stationary and mobile phases for a separation is the critical step for obtaining an acceptable separation.

Having chosen a separation mode the separation system must be optimized [375,550-555]. This usually begins by defining the important experimental variables and their range (parameter space) and selecting an appropriate optimization strategy to locate either an optimum or acceptable separation within this parameter space. Based on the retention range of the sample components either an isocratic (section 4.4.5) or gradient elution (section 4.4.6) separation may be employed. For simple mixtures it is recommended to first vary such parameters as the volume fraction of strong solvent, temperature and solvent type either alone or in combination. Effective optimization of selectivity by simultaneous control of more than one variable requires the selection of variables with a low correlation. Theory shows that changes in effective selectivity requires a difference in two or more solute dependent retention interactions that are different for the altered conditions.

The modest range of theoretical plates available in liquid chromatography makes efficiency optimization less effective than selectivity optimization. The peak capacity in isocratic liquid chromatography is about 50 with a separation time of 20 minutes and no more than about 250 with a separation time of 2 hours [555,556]. Without extensive optimization there is a reasonable probability of being able to baseline resolve about 7 compounds rising to 13-15 compounds after selectivity optimization. Similar results are found for gradient elution with simultaneous optimization of gradient time and temperature. For more complex samples the probability of success for a one-dimensional separation is low and mixtures containing more than 15 relevant components are regarded as difficult to separate.

Once the selectivity is optimized, a system optimization can be performed to improve resolution or to minimize the separation time. Unlike selectivity optimization, system optimization is usually predictable, since only kinetic parameters are generally considered. Typical experimental variables include column length, particle size, flow rate, instrument configuration, sample injection size, etc. Many of these parameters are connected to the chromatogram through reliable equations, and therefore, computer simulation approaches have been successful in providing a structured approach to this problem [375,557,558].

The final step in the method development process is validation [559-564]. Full validation involves statistical testing of various aspects of method performance (e.g. accuracy, precision, sensitivity, specificity, detection and quantification limits, linearity,

Table 4.13

Physical properties of some common solvents for liquid chromatography

Solvent	Boiling point (°C)	Viscosity (cP)	Refractive index	UV cutoff (nm)	Dielectric constant	Surface tension (dyne/cm)
Hexane	65	0.30	1.375	195	1.89	18.4
Toluene	111	0.59	1.497	284	2.4	
Diethyl ether	35	0.24	1.352	218	4.30	17.1
Methyl <i>t</i> -butyl ether	55	0.27	1.369	210	4.30	19.4
Tetrahydrofuran	66	0.55	1.407	212	7.58	26.4
Ethyl acetate	77	0.45	1.372	256	6.02	23.8
Chloroform	61	0.57	1.446	245	4.81	27.2
Dichloromethane	40	0.44	1.424	233	9.08	28.1
Acetone	56	0.36	1.359	330	20.7	23.3
Acetonitrile	82	0.36	1.344	190	37.5	19.1
Methanol	65	0.55	1.328	205	33.6	22.6
2-Propanol	82	2.40	1.377	205	18.3	21.8
Acetic acid	118	1.30	1.372		6.15	27.8
Water	100	1.00	1.333	<190	80.4	73.0

etc.) to ensure that the method is fit for its intended purpose. The extent of method validation should be appropriate for the scope of the method. For example, if it is intended to analyze a few samples by a single method without changing the operating conditions, then so long as the method is accurate, sensitive enough, and repeatable, that would probably be sufficient. On the other hand, if a method is to be used in other laboratories with different equipment, columns and operators, etc., the robustness of the method will be of equal importance. This would include the effects of changing parameters away from the determined optimum values and describing the effect on the performance of the method. A method is robust if small changes to critical parameters give consistently the same method performance. A method that is not robust requires further optimization before it can be considered fit for its intended purpose. The validation tests may indicate those parameters that are responsible for inadequate method performance and thus provide useful insight into how to re-optimize the procedure. In industries regulated by local or international agencies formal validation guidelines are available and enforced by the regulator. There are also a large number of software products to assist in selecting statistical tests for method validation.

4.4.1 Solvent Selection for Liquid Chromatography

Some broad generalizations can be made about the selection of preferred solvents for liquid chromatography. Suitable solvents have a low viscosity, are compatible with the detection system, are available in a pure form, are relatively inexpensive, and if possible, have low flammability and toxicity. In practice, any useful solvent must be able to dissolve the sample without reacting with it chemically. An abbreviated list of suitable solvents for liquid chromatography and their physical properties is given in Table 4.13 [565-568]. Special grades of solvents are available for HPLC; most are carefully purified

to remove contaminants, UV-absorbing impurities and particulate matter. In general, solvent impurities cause a drift in the detector baseline and diminished sensitivity under isocratic conditions, and large baseline fluctuations and spurious interfering peaks when gradient elution is used. Some solvents contain a preservative or antioxidant purposefully added to a solvent, for example the UV-absorbing 2,6-di-*tert*-butyl-4-methylphenol added to ethers, to minimize the formation of peroxides. The presence of preservatives can influence the chromatographic properties or detector compatibility of a solvent, restricting its general use. Some solvents are available with preservative, without preservative but with a limited shelf life, or with different preservatives not in conflict with specific applications.

Since detection in HPLC occurs on-line, compatibility with the detection system is very important. Low-wavelength UV detection compatibility is indicated by the UV cutoff. This property is often misinterpreted. The UV cutoff is the wavelength at which the absorbance of the solvent against air in 1-cm matching cells is equal to unity. It does not indicate the lowest wavelength at which solvent absorption is acceptable for measurements, and since the absorption edge is not vertical, it does not indicate the lowest wavelength at which solvent absorption will not interfere with the detection process. At wavelengths significantly longer than the cutoff detection should be straightforward. Solvents of low viscosity are preferred because they enhance column performance and minimize the column pressure drop for a given column length and/or particle size.

The solvation properties of liquids have been categorized according to a number of general schemes [568-572]. For chromatographic applications various empirical estimates of a solvent's strength and selectivity are widely used. The solvent strength is a single parameter estimate of the solvent's ability to cause migration in a chromatographic system. It is a composite property of the stationary phase and solvent and cannot be considered a fundamental property of the solvent alone. For example, water is considered a strong solvent in liquid-solid chromatography and a weak solvent in reversed-phase chromatography. Solvent selectivity is seen as the factor that distinguishes individual solvents that have suitable solvent strength for a separation. It is determined by the relative capability of the solvent to enter into specific intermolecular interactions, such as orientation or hydrogen bonding. Solvents can have similar solvent strength but different selectivity resulting in substantial differences in the separation order for sample components.

For chromatographic applications, the most useful models of solvent properties are the solubility parameter concept, Snyder's solvent strength and selectivity parameters, solvatochromic parameters and the system constants of the solvation parameter model for gas to liquid transfer. The Hildebrand solubility parameter, δ_H (total solubility parameter), is a rough measure of solvent strength, and is easily calculated from the physical properties of the pure solvent. It is equivalent to the square root of the solvent vaporization energy divided by its molar volume. The original solubility parameter concept was developed from assumptions of regular solution behavior in which the principal intermolecular interactions were dominated by dispersion forces.

This approach has been extended to polar solvents by decomposing the total solubility parameter into a series of polar partial solubility parameters that are treated as additive quantities [573,574]. The polar partial solubility parameters are only approximate, since there is no general agreement as to the correct method for their calculation. Current use of the solubility parameter model is generally limited to studies of the solubility of polymers, where the solubility parameters are useful for predicting solvents for size-exclusion and gradient polymer elution chromatography. A good solvent for a polymer has the same total solubility parameter as the polymer.

The solvent triangle classification method of Snyder is the most enduring approach to solvent characterization used by chromatographers, but in several respects is not entirely satisfactory [568,575,576]. Snyder classified solvents based on their interactions with three prototypical solutes determined by their gas-liquid distribution constants corrected for differences in solvent size, polarizability and dispersion interactions (assumed identical to the interactions of a hypothetical *n*-alkane with the same molar volume). Each value was then corrected empirically to give a value of zero for the polar distribution constant for saturated hydrocarbon solvents. Snyder chose the solutes nitromethane, ethanol and dioxane as probes for a solvent's capacity for dipole-type, hydrogen-bond base and hydrogen-bond acid interactions, respectively. The sum of the three polar distribution constants provides a measure of the solvent strength (P') and the ratio of the individual polar distribution constants to their sum a measure of selectivity (x_n , x_e and x_d). Representing each solvent by the three solvent selectivity coordinates and plotting the results on the surface of a triangle ($x_n + x_e + x_d = 1$) resulted in the classification of solvents into eight selectivity groups, Table 4.14. The trialkylamines are arbitrarily placed in group I although they are significantly more hydrogen-bond basic than the other solvents in this group. Chloroform, a moderately strong hydrogen-bond acid, is only loosely assigned to group VIII where the other members of the group consist of stronger hydrogen-bond acids, such as phenols and fluorine-containing alcohols. Solvents in the same group are expected to show similar separation properties and are only appropriate for fine tuning separations. Solvents of similar strength from different groups have different selectivity and are capable of providing changes in the separation order. The important contribution made by the solvent selectivity triangle approach was the idea that method development should involve the selection of a typical solvent (or a few solvents only) from each group. All groups should be represented in the method development process to ensure that the full range of available solvent selectivity is explored.

The most significant limitation of the solvent selectivity triangle approach is the association of an individual intermolecular interaction with the properties of a single solute. It is clear that the solubility of the prototypical solutes is due to multiple interactions [577]. The solubility of ethanol results from a composite of solvent dipolarity and hydrogen-bond basicity and acidity; for dioxane from solvent dipolarity and hydrogen-bond acidity; and for nitromethane predominantly from solvent dipolarity with small contributions from hydrogen-bond basicity and acidity. If it is assumed that the solubility of ethanol is predominantly due to its hydrogen-bond acidity then an inflated value for the hydrogen-bond basicity of the solvent is obtained. Even

Table 4.14

Solvent strength and selectivity parameters based on Snyder's selectivity triangle.

(S_i is an empirical solvent strength parameter for reversed-phase chromatography)

Solvent	Selectivity group	Solvent strength		Solvent selectivity		
		(P')	(S_i)	x_e	x_d	x_n
n-Butyl Ether	I	2.1		0.44	0.18	0.38
Diisopropyl Ether		2.4		0.48	0.14	0.38
Methyl <i>t</i> -Butyl Ether		2.7				
Diethyl Ether		2.8		0.53	0.13	0.34
n-Butanol	II	3.9		0.59	0.19	0.25
2-Propanol		3.9	4.2	0.55	0.19	0.27
1-Propanol		4.0		0.54	0.19	0.27
Ethanol		4.3	3.6	0.52	0.19	0.29
Methanol		5.1	3.0	0.48	0.22	0.31
Tetrahydrofuran	III	4.0	4.4	0.38	0.20	0.42
Pyridine		5.3		0.41	0.22	0.36
Methoxyethanol		5.5		0.38	0.24	0.38
Dimethylformamide		6.4		0.39	0.21	0.40
Acetic Acid	IV	6.0		0.39	0.31	0.30
Formamide		9.6		0.38	0.33	0.30
Dichloromethane	V	4.3		0.27	0.33	0.40
1,1-Dichloroethane		3.5		0.30	0.21	0.49
Ethyl Acetate	VI	4.4		0.34	0.23	0.43
Methyl Ethyl Ketone		4.7		0.35	0.22	0.43
Dioxane		4.8	3.5	0.36	0.24	0.40
Acetone		5.1	3.4	0.35	0.23	0.42
Acetonitrile		5.8	3.1	0.31	0.27	0.42
Toluene	VII	2.4		0.25	0.28	0.47
Benzene		2.7		0.23	0.32	0.45
Nitrobenzene		4.4		0.26	0.30	0.44
Chloroform	VIII	4.3		0.31	0.35	0.34
Dodecafluoroheptanol		8.8		0.33	0.40	0.27
Water		10.2	0	0.37	0.37	0.25

solvents with limited hydrogen-bond basicity could be classified as moderately strong hydrogen-bond bases due to the capacity for dipole-type interactions. Since it is impossible to find a prototypical solute that is a strong hydrogen-bond acid or base and is not dipolar, it is also impossible to characterize intermolecular interactions based on the solubility properties of single solutes. Linear solvation energy relationships, such as the solvatochromic and solvation parameter models, have proven more useful for identifying and quantifying the relative contribution of specific intermolecular interactions to the solvation process.

Table 4.15

Solvatochromic solvent selectivity parameters.

(Italicized solvents are only weakly attached to a group. A ? indicates that the value is unknown or uncertain)

Solvent	Solvatochromic parameters		
	π_1^*	α_1	β_1
n-Butyl Ether	0.27	0	0.46
Diisopropyl Ether	0.27	0	0.49
Diethyl Ether	0.27	0	0.47
(Triethylamine)	0.14	0	0.71)
Pyridine	0.87	0	0.64
Dimethylformamide	0.88	0	0.69
Dimethyl sulfoxide	1.00	0	0.76
(Nitrobenzene)	1.01	0	0.39)
Dichloromethane	0.82	0.30	0
(Chloroform)	0.58	0.44	0)
Ethyl Acetate	0.55	0	0.45
Methyl Ethyl Ketone	0.67	0.06	0.48
Dioxane	0.55	0	0.37
Acetone	0.71	0.08	0.48
Tetrahydrofuran	0.58	0	0.55
(Acetonitrile)	0.75	0.19	0.31)
Toluene	0.54	0	0.11
Benzene	0.59	0	0.10
(1,1-Dichloroethane)	0.81	0	0)
n-Butanol	0.47	0.79	0.88
2-Propanol	0.48	0.76	0.95
1-Propanol	0.52	0.78	?
Ethanol	0.54	0.83	0.77
(Methanol)	0.60	0.93	0.62)
Acetic Acid	0.64	1.12	?
Formamide	0.97	0.71	?
Water	1.09	1.17	0.18
(2,2,2-Trifluoroethanol)	0.73	1.51	0)

The solvatochromic parameters are derived from spectroscopic and other measurements specifically designed to measure only a single interaction. In addition, the values are averages of the results from several solutes for each parameter and somewhat independent of solute identity. The most comprehensive solvatochromic treatment of solvent selectivity are the π_1^* , α_1 and β_1 parameters of Kamlet and Taft, Table 4.15 [568-570, 578]. The π_1^* value is an index of solvent dipolarity/polarizability, normalized to dimethyl sulfoxide = 1. The α_1 scale of hydrogen-bond acidity measures the

ability of a solvent to donate a proton in a solvent-solute hydrogen bond, normalized to a value of 1.0 for methanol. The β_1 scale of hydrogen-bond basicity is a measure of the ability of the solvent to accept a proton (donate an electron pair) in a solvent-solute hydrogen bond, normalized to a value of 1.0 for hexamethylphosphoramide. By hierarchical cluster analysis the solvents can be categorized into groups with similar selectivity, as indicated in Table 4.15 [578]. The ethers are weakly dipolar and hydrogen bond basic solvents with no hydrogen-bond acidity. This group is loosely connected to triethylamine, which is a stronger hydrogen-bond base. The second group of solvents headed by pyridine are stronger dipolar/polarizable and hydrogen-bond base solvents than the ethers with no hydrogen-bond acidity. Nitrobenzene is only weakly connected with this group and is a much weaker hydrogen-bond base. Dichloromethane and chloroform are grouped together but with obvious differences. They possess intermediate dipolarity/polarizability and hydrogen-bond basicity with no or weak hydrogen-bond acidity. The large group of solvents headed by ethyl acetate possess intermediate dipolarity/polarizability and hydrogen-bond basicity with no or weak hydrogen-bond acidity. Acetonitrile is loosely connected to this group but is really behaving independently. Toluene and benzene have intermediate dipolarity/polarizability and weak hydrogen-bond basicity and no hydrogen-bond acidity. The alcohols are strong hydrogen-bond acids and bases with intermediate dipolarity/polarizability. Water and 2,2,2-trifluoroethanol are only loosely grouped together and differ from the other solvents in being strong hydrogen-bond acids with minimal hydrogen-bond basicity.

The $E_T(30)$ scale of solvent polarity is based on the position of the intermolecular charge transfer absorption band of Reichardt's betaine dye [570-572,579]. $E_T(30)$ values are available for several hundred common solvents as well as a number of binary solvent mixtures. It is also known that solvent $E_T(30)$ values are adequately represented by a linear combination of π_1^* and α_1 solvatochromic parameters. Its main use in chromatography has been as a single parameter estimate of solvent strength for the prediction of retention in reversed-phase chromatography [299,580,581]. Initial reports that plots of the logarithm of the retention factor against $E_T(30)$ values for aqueous organic mobile phases show greater linearity than similar plots against the volume fraction of organic solvent are not well founded when extended composition ranges are employed. In addition, $E_T(30)$ values do not measure the polarity of bulk solvent mixtures, but rather the local polarity of the solvent shell surrounding the betaine dye, which because of selective solvation, is not necessarily identical to the bulk solvent composition. For these as well as other reasons the $E_T(30)$ solvent polarity scale is not as widely used in chromatography as it is in other areas of chemistry.

The main problem with solvent classification schemes based on the solvatochromic parameters is that it considers only the polar interactions of the solvents and not their cohesive energy [578,582]. The transfer of solute from one solvent to another occurs with (approximate) cancellation of dispersion interactions, but the energy required for cavity formation in the two solvents is not necessarily self-canceling, and when one of these solvents is water, cancellation of the cavity term is unlikely. Solvent classification schemes needs to consider the cohesive energy of the solvent as well as its capacity

Table 4.16

System constants from the solvation parameter model for transfer from the gas phase to the solvent at 25°C

Solvent	System constants					
	<i>r</i>	<i>s</i>	<i>a</i>	<i>b</i>	<i>l</i>	<i>c</i>
Benzene	-0.31	1.05	0.47	0.17	1.02	0.11
Toluene	-0.22	0.94	0.47	0.10	1.01	0.12
Chlorobenzene	-0.55	1.25	0.36	0	1.04	0.05
Chloroform	-0.60	1.26	0.28	1.37	0.98	0.17
1,2-Dichloroethane	-0.15	1.44	0.65	0.74	0.94	0.01
Hexane	-0.17	0	0	0	0.98	0.29
Cyclohexane	0	-0.18	0	0	1.02	0.22
Carbon tetrachloride	-0.20	0.35	0.07	0.27	1.04	0.23
Methanol	-0.22	1.17	3.70	1.43	0.77	0
Ethanol	-0.21	0.79	3.63	1.31	0.85	0.01
Octan-1-ol	-0.12	0.44	3.69	0.59	0.93	0.07
Propan-1-ol	-0.19	0.65	4.02	1.04	0.87	-0.03
Acetonitrile	-0.22	2.19	2.38	0.41	0.73	0
Dimethyl sulfoxide	-0.20	2.89	5.46	0	0.73	-0.59
Water	0.82	2.74	3.90	4.80	-2.13	-1.27
2,2,2-Trifluoroethanol	-0.61	1.46	1.90	4.46	0.63	-0.13

for polar interactions. The solvation parameter model, in the form suitable for solute transfer from the gas phase to a liquid (section 1.4.3) meets this requirement. Solvent properties are characterized by their capacity for specific interactions by individual system constants. The *l* system constant together with contributions contained in the model constant (*c* term) accounts for the contributions from cavity formation and dispersion interactions to the solvation process. The other system constants account for the capacity of the solvent for electron lone pair interactions (*r*), dipole-type interactions (*s*), and solvent hydrogen-bond acidity (*b*) and hydrogen-bond basicity (*a*). For solvents of interest to liquid chromatography the system constants for the limited data available are summarized in Table 4.16 [578]. The solvents can be grouped according to their similarity by hierarchical cluster analysis, Figure 4.22. The group headed by benzene has low cohesion, moderate dipolarity/polarizability and weak hydrogen-bonding properties. Chloroform and 1,2-dichloroethane have low cohesion but are quite dipolar and hydrogen-bond acidic with low hydrogen-bond basicity. The hydrocarbon solvents and carbon tetrachloride have low cohesion and a minimal capacity for polar interactions. The alcohols are quite cohesive, strongly hydrogen-bond basic and significantly dipolar/polarizable and hydrogen-bond acidic. The remaining polar solvents are classified as behaving independently. Acetonitrile is quite cohesive, strongly dipolar and hydrogen-bond basic, and weakly hydrogen-bond acidic. Water is the most cohesive of the solvents and the strongest hydrogen-bond acid. It is also quite dipolar and hydrogen-bond basic. Its properties clearly set it apart from the other

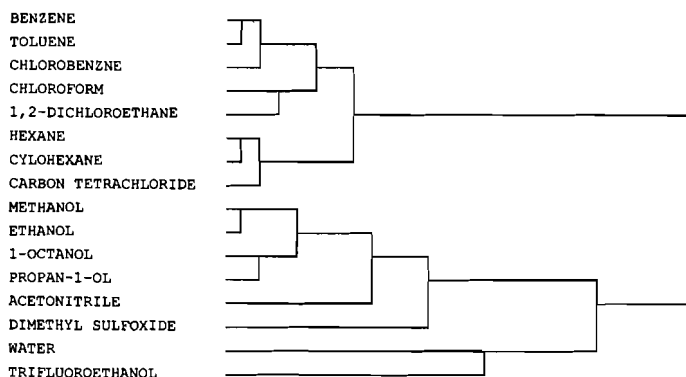


Figure 4.22. Complete linkage dendrogram for solvents with the system constants of the solvation parameter model (Table 4.16) as variables.

solvents in Table 4.16. 2,2,2-Trifluoroethanol is almost as hydrogen-bond acidic as water but is less cohesive, dipolar/polarizable and hydrogen-bond basic.

4.4.1.1 Solvent Mixtures

Few separations by liquid chromatography are performed using single solvents because of the limited possibilities offered for the simultaneous optimization of solvent strength and selectivity. Binary and higher order solvent mixtures provide a more logical and flexible solution to this problem. Mixing a strength-adjusting solvent with various volume fractions of a strong solvent enables the complete solvent strength range between the extremes represented by the pure solvents to be covered. The strength-adjusting solvent is usually a non-selective solvent, such as water for reversed-phase chromatography and hexane for liquid-solid chromatography. The solvent strength (S_T) of a mixed mobile phase is the arithmetic average of the solvent strength weighting factors (S_i) adjusted according to the volume fraction of each solvent (ϕ_i). For evaluation this is expressed as $S_T = \sum_i S_i \phi_i$ with Snyder's polarity index, P' , and S_i values for pure solvents (Table 4.14) taken as the solvent weighting factors for normal-phase and reversed-phase separation systems, respectively. For a binary solvent mixture containing 60% methanol and 40% water used in reversed-phase chromatography, the solvent strength of the mixed solvent is calculated as follows

$$S_T = S_m \phi_m + S_w \phi_w = (2.6)(0.60) + (0)(0.40) = 1.56$$

where the subscripts m and w identify the parameters for methanol and water, respectively.

Several mobile phase optimization strategies in liquid chromatography are based on the use of isoeluotropic solvents, that is, solvent mixtures of identical strength but different selectivity. Suitable binary (and higher order) solvent mixtures are usually selected based on their location in the solvent selectivity triangle (Table 4.14). To

maximize the differences in selectivity, solvents are selected from different selectivity groups that lie close to the triangle apexes. The selected solvents must also be miscible with the strength adjusting solvent. For example, for reversed-phase chromatography a suitable selection might be methanol (group II), acetonitrile (group VI) and tetrahydrofuran (group III) mixed with water to control solvent strength. For liquid-solid chromatography methyl *tert*-butyl ether (group I), chloroform (group VIII) and dichloromethane (group V) mixed with hexane to control solvent strength.

The composition of isoeluotropic solvents is simply calculated using the additive nature of the volume fractions and their respective solvent weighting factors. If, the solvent strength of the methanol-water (60:40) mixture was about optimum for a particular separation but not its selectivity, the band spacing (resolution) could be further optimized by using isoeluotropic mixtures containing water-acetonitrile and water-tetrahydrofuran. The composition of these mixtures is calculated as indicated for acetonitrile-water

$$1.56 = S_a \phi_a + S_w \phi_w = 3.2 \phi_a + (0) \phi_w$$

$$\text{and } \phi_a = 1.56/3.2 = 0.49 \text{ or } 49\%(\text{v/v})$$

where the subscript a refers to acetonitrile. Thus a mixture of acetonitrile-water (49:51) has similar solvent strength to a mixture of methanol-water (60:40). In a similar manner a mixture of tetrahydrofuran-water (35:65) is isoeluotropic with the two previous binary solvent mixtures.

Alternatively, the following empirical relationships can be used to estimate the equivalent solvent composition of isoeluotropic binary solvent mixtures given that experimental conditions have been established using methanol as the organic modifier in reversed-phase liquid chromatography

$$\phi_a = -0.49\phi_m^3 + 0.953\phi_m^2 + 0.447\phi_m$$

$$\phi_t = -0.42\phi_m^3 + 0.702\phi_m^2 + 0.433\phi_m$$

where ϕ_a and ϕ_t are the volume fractions of acetonitrile and tetrahydrofuran required to prepare a binary solvent mixture with water equivalent in strength to a given volume fraction of methanol, ϕ_m [583]. Since the separation time for a mixture is dependent on the elution time for the last component to leave the column, any exceptional behavior of this compound will dramatically influence the calculation of the solvent strength to elute the sample in an acceptable time. All the above approaches have to be considered approximate and capable of producing unsatisfactory results for some solutes or conditions.

The viscosity, and other colligative properties of solvent mixtures, depends on the relative proportion of the individual solvents. If the mixtures are nearly ideal then the viscosity can be approximated by the sum of the products of each solvent's

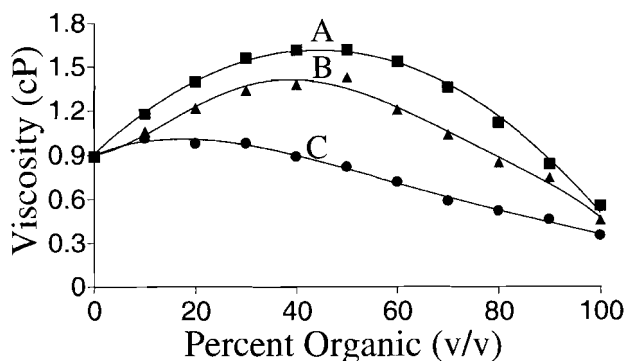


Figure 4.23. Variation of viscosity with composition for binary mixtures of water and tetrahydrofuran (A), methanol (B) and acetonitrile (C).

viscosity and its mole fraction. Many solvent mixtures, particularly those containing water, exhibit non-ideal behavior. The viscosity at 25°C for typical reversed-phase binary solvent mixtures is shown in Figure 4.23. The viscosity of water-methanol and water-tetrahydrofuran mixtures is particularly unfavorable for middle composition mixtures compared with water-acetonitrile mixtures. Water-2-propanol mixtures have even higher viscosities. Higher viscosity results in a small reduction in column efficiency, but more importantly, higher column operating pressures (section 1.5.1).

Binary solvent mixtures provide a simple means for controlling solvent strength but only limited opportunities for controlling solvent selectivity. With ternary and quaternary solvent mixtures it is possible to fine tune solvent selectivity while maintaining a constant solvent strength. In addition, there are only a small number of organic modifiers that can be used as binary mixtures with water. Many more mobile phases of the same solvent strength can be prepared if ternary and quaternary solvent systems are used. An example of mobile phase selectivity optimization using a ternary solvent mixture is shown in Figure 4.24 [584]. The separation in Figure 4.24A was obtained with the binary mobile phase methanol-water (50:50). The solvent strength of this mixture is about correct for the separation based on the range or retention factors, but components 1 and 2 are not separated. This indicates that a change in selectivity is required. The separation obtained with the isoelutropic solvent mixture tetrahydrofuran-water (32:68), Figure 4.24B, shows a change in component resolution with a range of retention factors similar to Figure 4.24A. Components 1 and 2 are now well separated but components 2 and 3 are not. Mobile phase strength and selectivity are optimized in the ternary solvent system methanol-tetrahydrofuran-water (10:25:65), Figure 4.24C, in which all the sample components are adequately separated.

4.4.2 General Strategies

The low peak capacity of liquid chromatography makes selectivity optimization the most powerful approach to achieving the desired resolution in a chromatogram.

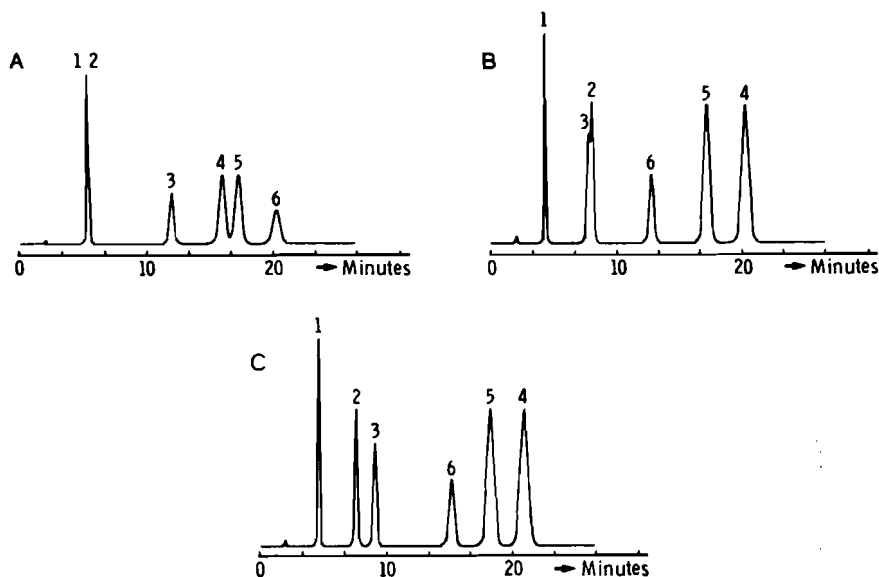


Figure 4.24. An example of the use of a ternary solvent system to control mobile phase selectivity in reversed-phase chromatography. A, methanol-water (50:50); B, tetrahydrofuran-water (32:68); and C, methanol-tetrahydrofuran-water (35:10:55). Identification: 1 = benzyl alcohol; 2 = phenol; 3 = 3-phenylpropanol; 4 = 2,4-dimethylphenol; 5 = benzene; and 6 = diethylphthalate. (From ref. [583]; ©Elsevier)

Selectivity can be optimized following a wide range of protocols varying from an intuitive trial and error approach to automated computer-aided methods [378,550-552,585-587]. However, no single approach has emerged as superior to all others and in practice, the expertise of the chromatographer and the availability of specific equipment and software usually dictate the approaches employed in individual laboratories.

The first step in developing an optimization strategy is to define the parameter space to be searched for an acceptable separation. The parameter space is that combination of experimental variables and their limiting values that define the search area. An acceptable separation must be within the parameter space, otherwise the search techniques will only result in failure. On the other hand the parameter space should not be set artificially large, since the search techniques will become inefficient. The parameter space is reduced as much as possible by including only those variables which have a significant effect on the selectivity and by assigning sensible limits to each variable. The number of significant variables that affect selectivity is a matter of judgment, although factorial designs can be used to identify those variables contributing most to selectivity. In most cases a few preliminary scouting experiments are used to define the limits for the experimental variables.

The parameter space is next searched for a global optimum by performing a series of separations according to a formal experimental design. The number of separations

depends on the chosen experimental design. Three broad strategies are commonly employed for isocratic separations. Simultaneous or grid search methods are used to model the quality of the separation throughout the parameter space involving the collection of data at predefined points followed by inspection of the chromatograms to define the optimum separation conditions. In sequential methods a small number of initial experimental results are used to direct the chromatographer to conditions where better results are expected, for example, simplex optimization. The third type of search technique is based on iterative and interpretive methods, in which the object is to model the retention surface for each sample component on the basis of experimental retention data. These methods are perhaps the most popular because of the small number of required experiments, typically 7-10. Solute recognition, however, is essential and the accuracy of any predictions depends on the model used to describe the response surface.

Interpretive methods require the recognition of individual peaks in each chromatogram so that regression equations can be fitted to the model used to define the response surface. Peak recognition can be achieved by injecting individual standards for each solute in each chromatogram, from a comparison of peak areas or peak ratios, from UV spectra, or from characteristic mass ions obtained by mass spectrometric detection. Injecting all solutes individually is not only slow but also infeasible for samples of unknown composition. The accuracy of peak area and peak ratio methods is limited where there are large differences in component concentrations and by changes in detector response and baseline noise associated with solvent changes. In addition, peak assignments based on relative areas will not reveal the real number of components present. A method to determine whether all sample peaks are eluted from the column is also required. The recording of full UV spectra with a photodiode array detector or characteristic mass detection together with chemometric data analysis, is the preferred approach to this problem. The success of even these methods, however, depends on the resolution, the number of components, detector noise, the spectral similarity and the relative amounts of the components. Clearly, optimization is simplified when the exact number of components (or relevant components) in the mixture is known and standards are available for each component. For samples with an unknown composition, determining the total number of detectable components is difficult, particularly if individual components are present in significantly different amounts, but is also relevant to the optimization process, as otherwise it is impossible to state with absolute certainty when a separation is complete.

If the selectivity optimization is carried out manually, then the quality of the separation can be evaluated by visual inspection of the chromatograms. This approach, though, is inadequate for automated optimization procedures, and the quality of a chromatogram must be expressed by a single-valued and easily calculated mathematical function, referred to as an objective function or chromatographic response function (section 1.6.2). The variation of the magnitude of this function over the parameter space is called the response surface. In the general case, the response surface resembles a mountain area with several peaks and valleys. The highest feature of the response surface represents the global optimum and defines that combination of experimental

parameters producing the best possible separation as determined by the objective function. Smaller features represent local optima expressing the best separation, as defined above, in a section of the parameter space, but an inferior separation with respect to the global optimum. The purpose of the search technique is to locate the global optimum and to distinguish it from inferior local optima.

4.4.3 Selection of Gradient or Isocratic Methods

In reversed-phase chromatography method development is often begun with a gradient elution separation. From this separation it is possible to determine whether isocratic or gradient elution is the appropriate technique for a given sample and to estimate either the solvent strength for isocratic separations or the gradient range for gradient elution [583,588-594]. This approach is based on the theory of linear solvent strength gradients (section 4.4.6).

An initial linear gradient of 5-100% acetonitrile or methanol at a rate of increase that depends on the column hold-up time (e.g. $t_G / t_M \approx 15$, where t_G is the gradient run time and t_M the column hold-up time) is employed for the trial separation. If the ratio of the difference in retention time between the first detected peak (t_{g1}) and the last detected peak (t_{gn}) during the gradient exceeds about 25% of the gradient time ($[t_{gn} - t_{g1}] / t_G > 0.25$), it is unlikely that a suitable isocratic method can be developed for this sample. Or, if the percent strong solvent composition for the first and last peak to elute during the gradient exceeds 15% then an isocratic separation is unlikely. In both cases the wide range of isocratic retention factors for the sample makes gradient elution the preferred separation technique.

If the scouting gradient indicates that an isocratic separation is possible then the gradient retention time can be used to estimate the solvent strength for the isocratic separation. All of these methods are based on the assumption of a linear retention model for isocratic elution of the form $\log k = \log k_w - S\phi$ with values for k_w and S for each solute estimated from gradient parameters. The S value is assumed to be independent of solute structure, which is only a rough approximation (section 4.3.1.1). The retention time for the last eluting peak is often used to fix the range of retention factors for the isocratic separation by assigning a value of $k \approx 10$ for a simple separation or $k \approx 20$ for a more complex mixture. This value can be refined later to minimize the separation time. Alternatively, the binary solvent mixture used to elute the first detected peak with $k = 1$ and the last detected peak with $k = 10$ can be calculated. The optimum isocratic solvent strength will lie between these two values. The calculation methods are summarized in Table 4.17. Some of the calculations are rather tedious in the absence of appropriate software. Computer-assisted approaches allow simulation of chromatograms with estimated positions for all sample components.

If two gradient runs are used, in which only the gradient time is varied (e.g. 20 and 60 min), the resulting retention data can be used to predict retention as a function of mobile phase composition in the corresponding isocratic separations. Linear plots of $\log k$ against mobile phase composition can be constructed. In those cases where

Table 4.17

Estimation of the optimum solvent strength for isocratic separations from gradient elution

(i) Single binary solvent gradient

Retention time for a solute with an optimum retention factor value

$$t_k = t_A - 2t_M - t_D$$

 t_A = average elution time $(t_{g1} - t_{g1}) / 2$ based on the first and last peak eluted during the gradient; t_M = column hold-up time; and t_D = gradient delay time.Isocratic solvent composition corresponding to t_k

$$\%B = (\text{initial } \%B) + (t_k / t_G)(\text{final } \%B - \text{initial } \%B)$$

 $\%B$ = percent strong solvent for the isocratic separation using a binary mobile phase; initial $\%B$ = percent strong solvent at the start of the gradient; final $\%B$ = percent strong solvent at the end of the gradient; and t_G = gradient run time.*(ii) Single binary solvent gradient (acetonitrile-water)*Let $T_R = t_g - t_M - t_D$ and $c = b / t_M$

$$k_0 = (10^{cT_R} + 2.3ct_D - 1) / 2.3ct_M$$

$$\log k_W = \log k_0 + 0.042 (\text{initial } \%B)$$

$$\log k = \log k_W - S\phi \text{ with } S = 4.2 (M < 500) \text{ or } S = 0.48M^{0.43} (M > 500)$$

 $(S = 3.6 \text{ is a better value for cyanopropylsiloxane-bonded phases in above relationships})$ t_g = gradient elution time; b = gradient steepness parameter; k_0 = isocratic retention factor for the solute in the starting solvent composition; k_W = estimated isocratic retention factor for water as the mobile phase; ϕ = volume fraction of strong solvent ($\%B / 100$); and M = solute molecular mass.*(iii) Two gradients with a difference of ≈ 3 -4 in gradient run times (t_G)*The gradient retention time, t_g , for each solute in the two gradients is entered into

$$t_g = (t_M / b) \log [2.3k_0b + 1] + t_M + t_D$$

Equation is solved numerically to give values of k_0 and b From which values of k_W and S are determined

$$\log k = \log k_W - S\phi$$

$$S = b t_G / \Delta\phi t_M$$

$$\log k_W = \log k_0 + S (\text{initial } \%B / 100)$$

 $\Delta\phi$ = gradient range (final $\%B$ - initial $\%B$)

the individual slopes (S values) are different, or if the slopes are the same but the intercepts (k_W) are different, solvent strength optimization can be employed to change band spacing. Method development, aided by computer simulation, will usually be faster with this approach than solvent selectivity optimization with different mobile phases. The overall approach, however, is less powerful because changes in band spacing accompanying changes in solvent strength are not as large as those observed by changing mobile phase solvents. The data can be plotted as relative resolution maps; a plot of the resolution between adjacent bands exhibiting the lowest resolution for a column with a constant plate number as a function of the binary mobile phase composition. These plots resemble window diagrams (section 2.4.2) and are interpreted in a similar manner. This approach appears particularly promising for separating simple mixtures containing components that differ in molecular weight or functionality.

The solvent strength of binary solvents providing adequate retention can also be estimated from a series of sequential isocratic chromatograms of decreasing solvent

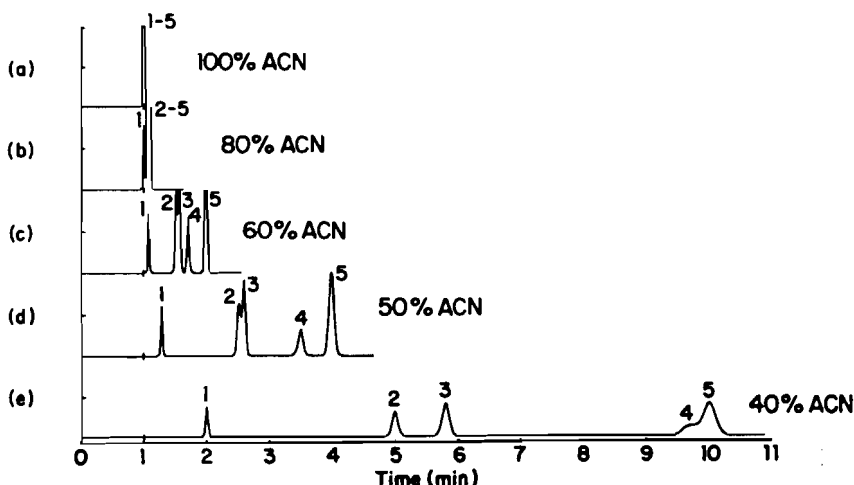


Figure 4.25. Sequential, isocratic elution using a stepwise reduction in solvent strength to identify a binary solvent of acceptable strength for elution of a five component mixture.

strength, Figure 4.25. Scouting starts with the strongest solvent first (acetonitrile), followed by subsequent separations using binary solvents containing increasing amounts of the strength adjusting solvent (water), initially added in 20% (v/v) increments. The column hold-up time in this example is 1 minute and the desired retention time for the last peak is 11 min ($k = 10$), corresponding to a binary mobile phase containing 40% (v/v) acetonitrile. The original step size of 20% (v/v) may have to be reduced in the region of the desired retention range to obtain the optimum value for the solvent strength.

4.4.4 Simultaneous Optimization of Two Variables

It is generally recognized that the simultaneous optimization of two separation variables is a more effective approach for method development when the two variables are no more than weakly correlated and both affect selectivity. Typical examples are the gradient time or solvent strength (% strong solvent in a binary mixture) and temperature, solvent strength and pH, or solvent strength and ion-pair reagent concentration [595-598]. The efficient application of this approach requires a good experimental design and the use of computer simulations to predict the optimum separation conditions based on a relatively small number of experiments. The same argument can be made for multivariate optimization of three or more variables, but then the experimental designs are more complex and require a larger number of experiments and the computer simulations become more difficult to interpret [552]. Some simple examples of the latter approach are presented in the sections that follow this one.

Separations as a function of temperature and binary mobile solvent strength in reversed-phase chromatography can be optimized based on four initial experiments.

Either isocratic or gradient elution conditions are used where temperature and volume percent strong solvent or the gradient time are varied. Gradient separations are generally preferred, as there is then no need for trial-and-error experiments to adjust the retention factor range to acceptable values throughout the parameter space to obtain reliable computer simulations. Isocratic predictions from gradient separations are generally less accurate than gradient predictions. One additional experiment, however, whose conditions can be predicted by computer simulation, can be used to correct errors in the isocratic predictions. Typically, two gradient runs with different gradient times (e.g. $t_G = 20$ and 60 min.) are performed at two temperatures (e.g. 35°C and 70°C) with all other conditions constant. From the gradient retention times, $\log k_W$ and S are calculated for each solute at each temperature, as outlined in Table 4.17. This allows the prediction of the retention factor at these two temperatures for any value of the solvent strength. The coefficients of the linear relationship between the retention factor and temperature can then be determined as a function of solvent strength. This allows prediction of the retention factors as a function of temperature at any value of the mobile phase composition. The results are usually presented as a contour map of resolution with the variables temperature and volume percent strong solvent as the axis. The area on the plot with the highest resolution for the most difficult to separate pair is used as an estimate of the optimum separation conditions. A “reflection” procedure allows correction of the predicted optimum by determining the difference (error) between the predicted and experimental separations, followed by a subtraction of this error to arrive at the correct final prediction. The reflection procedure may suggest an adjustment to temperature, solvent strength or both to reproduce the predicted separation. The reflection procedure is usually successful when overlap of a critical band-pair or when two critical band-pairs merge into a triplet. When the optimization procedure does not lead to an adequate separation, the problem can usually be traced to one of two causes. The sample contains too many (twenty or more) components and the subsequent chromatogram is too crowded or the sample contains compounds of similar structure. In the former case, two separations of the sample with different optimized conditions, so that every solute is resolved adequately in one run or the other is probably the best option. In the later case, a different strong solvent or stationary phase will probably be required for the separation.

4.4.5 Search Strategies for Optimizing Isocratic Separations

Once the correct solvent strength for an acceptable range of retention factors has been established and band spacing optimized as far as possible by solvent strength optimization, further improvement in the separation is obtained by selectivity optimization. Selectivity optimization is usually achieved by varying either the mobile phase composition at a constant solvent strength or by changing the stationary phase [375,550-552,599]. Mobile phase optimization is more convenient and is explored first. Transfer rules are used to calculate the composition of isoeluotropic binary (or higher order) solvent mixtures with complementary selectivity (section 4.4.1.1). For reversed-phase

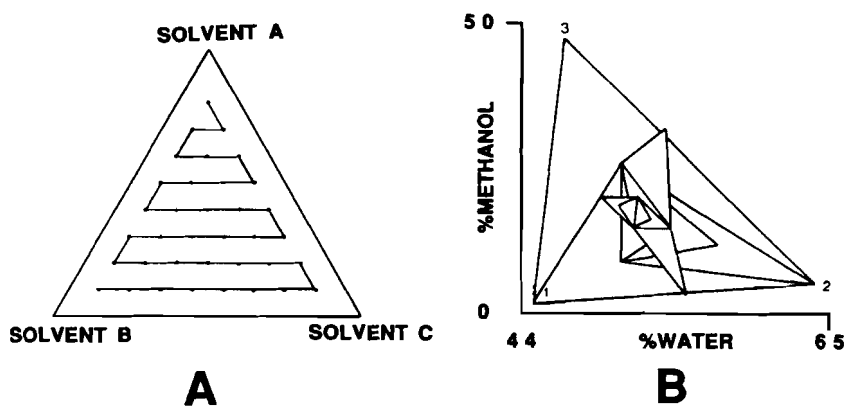


Figure 4.26. Experimental design for mobile phase composition optimization using a grid search (A) and simplex search (B).

chromatography the selectivity adjusting solvents are usually methanol, acetonitrile and tetrahydrofuran blended in different proportions with water as the strength adjusting solvent. The three isoeluotropic binary solvents can be placed at the apexes of a triangle whose boundaries define the parameter space. The parameter space contains all those ternary and quaternary mobile phases that can be produced by combining the three binary mobile phases at a constant solvent strength. Various experimental designs are then employed for an efficient search of the parameter space to optimize selectivity for the separation.

4.4.5.1 Direct Search Methods

Direct search methods include grid searches and simplex searches. These methods do not presume any model for the response surface and aim to map the whole parameter space at reasonably selected intervals to locate the best separation. The best separation is considered the optimum separation and if inadequate a new parameter, for example, a change in one of the binary solvents is required. Grid searches explore the parameter space in small steps according to a fixed experimental design, Figure 4.26 [551,552]. Chromatograms are recorded at each of the design points and the best separation is selected either visually or by maximizing an objective function. The method is easily automated and provides a complete picture of the response surface. The global optimum should be obtained but the number of experiments, which may include additional experiments for peak tracking, can be excessive, particularly for simple separations.

Sequential optimization strategies like simplex designs rely on the result from previous experiments to provide guidance and direction for additional experiments [551,552,600,601]. A simplex is defined as a geometric figure having one more vertex than the number of variables being optimized. Thus, a two variable simplex is a triangle and a three variable simplex a tetrahedron, etc. The optimization proceeds by rejecting the vertex that has the worst experimental response and the location of

the next data point is found by reflecting the simplex in the opposite direction. The direction of advance of the simplex depends solely on the ranking of responses by an objective function. Expansion and contraction of the simplex, as shown in Figure 4.26B, increases the search efficiency by allowing the simplex to expand and thus accelerate towards the optimum region. Having located it approximately, the simplexes contract and reduce the search region until the optimum is precisely located. The advantages of the simplex procedure are: it is able to optimize many interdependent variables with no prior knowledge of the mode of separation or sample complexity; peak tracking is not required; new variables can be introduced during the optimization process for the price of just one additional experiment per variable; and the progress of the optimization can be assessed at all times and terminated when an acceptable separation is obtained. The simplex has disadvantages as well. The success of the optimization process depends entirely on the quality of the objective function used to rank each experiment. The simplex does not handle irregular response surfaces very well. It may become stalled on a local optimum instead of proceeding to the global optimum. The simplex searches the response surface for the global optimum rather than mapping the response surface, so that at the end of the optimization procedure little insight into the shape of the response surface is obtained. Although in favorable cases the simplex will probably locate the global optimum with fewer experiments than for grid search methods, it still requires a relatively large number of experiments. A typical grid search might require about 100 experiments while for the simplex approach perhaps 25-40 would be required [602]. Although both methods can be fully automated, a typical chromatographic experiment is likely to require about 30 minutes or so for the separation and column re-equilibration steps, and therefore, neither method represents a fast approach to selectivity optimization.

4.4.5.2 Interpretive Methods

Interpretive methods involve modeling the retention surface (as opposed to the response surface) based on experimental retention data [375,550-552]. The model for the retention surface may be graphical or algebraic and based on mathematical or statistical theories. The retention surface is generally much simpler than the response surface and can be described by an accurate model on the basis of a small number of experiments, typically 7 to 10. Solute recognition in all chromatograms is essential, however, and the accuracy of any predictions is dependent on the quality of the model.

The mixture-design statistical approach has been widely used for selectivity optimization in reversed-phase chromatography [603-608]. After reduction of the parameter space to three isoelutotropic binary solvent mixtures with retention factors in the optimum range, a fixed experimental design is applied. This design consists of seven experiments. Separations in each of the three binary solvents which can be taken as the apexes of a triangle, separations in each of the three ternary solvent mixtures obtained by alternately mixing equal volumes of any two of the binary solvents (corresponds to the midpoint on each side of the triangle), and finally the quaternary solvent mixture obtained by mixing equal volumes of all three binary solvents (corresponds to the

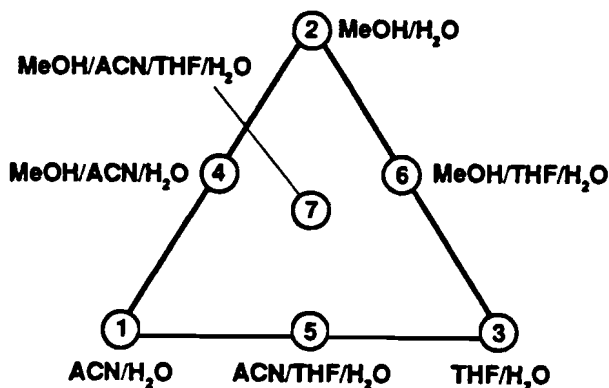


Figure 4.27. Experimental design for selectivity optimization in reversed-phase chromatography by the mixture-design technique. (From ref. [375]; ©John Wiley & Sons)

midpoint of the triangle), Figure 4.27. The retention factors obtained can be treated in several ways. A visual comparison of the seven chromatograms may indicate that a successful separation has been achieved already, for example Figure 4.28, or indicate a region of the parameter space where additional trial experiments could be expected to yield the desired result. This restricted area of the parameter space could be searched again using a smaller triangle with new apexes selected around the optimum region, for example from Figure 4.28, by choosing points 3, 5 and 6 or 4, 6 and 7 as the new apexes of the triangle. Although in Figure 4.28, the separation obtained at point 5 would likely be deemed acceptable and further optimization made superfluous, provided that it was known that the mixture contained only nine components. A second approach would be to construct a retention surface from the experimental data by fitting the data to a suitable model, for example, Eqs. (4.35) and (4.36)

$$\ln k = a_1 X_1 + a_2 X_2 + a_3 X_3 + a_4 X_1 X_3 + a_5 X_2 X_3 + a_6 X_1 X_2 X_3 \quad (4.35)$$

$$\ln k = a_1 X_1^2 + a_2 X_2^2 + a_3 X_3^2 + a_4 X_1 X_3 + a_5 X_2 X_3 + a_6 X_1 X_2 \quad (4.36)$$

where X_1 , X_2 and X_3 are the fractions of the pseudocomponents (the binary isoelectrostatic mixtures) and the a terms are coefficients evaluated for each solute using the observed values for the retention factors in the seven experiments with known values of X_1 , X_2 and X_3 defined by the experimental design. Likewise, the response surface can be fitted to Eqs. (4.35) or (4.36) by replacing the retention factor term with a suitable objective function. However, the general limitations of objective functions (section 1.6.2) and inherent problems with peak crossovers as the mobile phase composition is changed, have resulted in this approach being largely replaced by the use of overlapping resolution maps. The retention factors for each solute are predicted at all mobile phase compositions within the parameter space. A grid is constructed for which

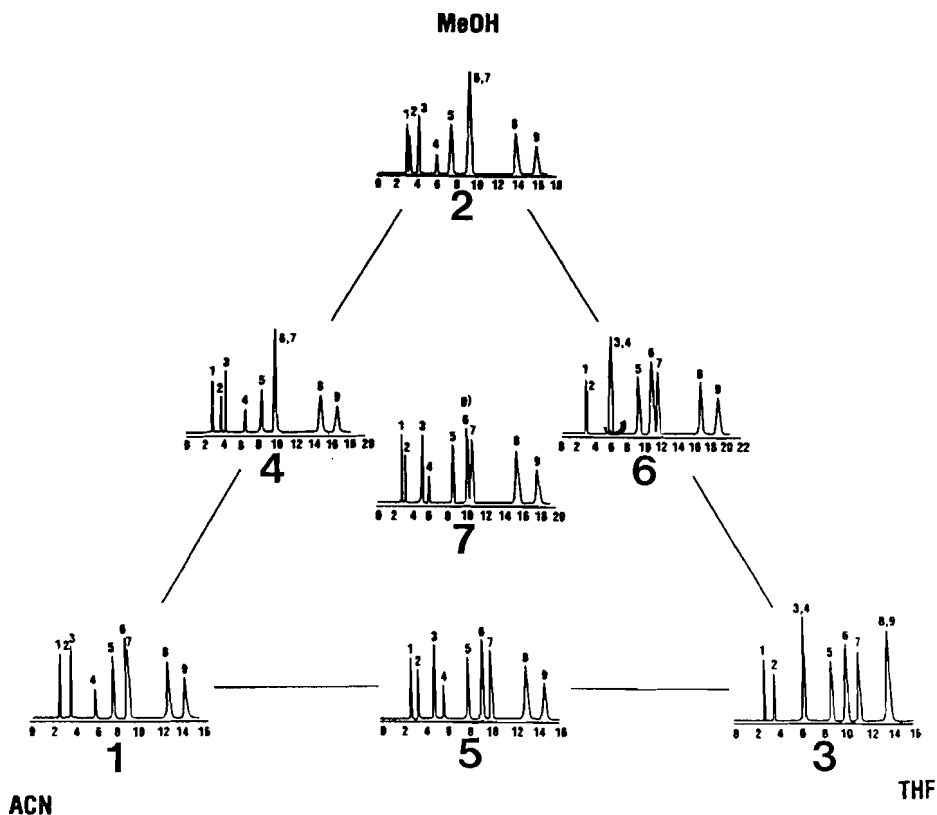


Figure 4.28. Separation of nine substituted naphthalene compounds using the mixture-design statistical technique to optimize the composition of the mobile phase (From ref. [375]; ©John Wiley & Sons).

the pseudocomponents (X) are changed by one percent at a time and at each mobile phase composition the resolution is predicted for all peak pairs. A resolution contour plot is prepared for each pair of components depicting the resolution for the pair in all solvent compositions. A desired minimum resolution (e.g. 1.5) between peak pairs is then specified and this enables areas on the contour plot to be eliminated as being of no further interest since they do not meet the desired resolution criterion. Figure 4.29 shows the peak-pair resolution maps for the nine substituted naphthalene compounds separated in Figure 4.28 with the solvent composition regions for each adjacent peak pair that fails to meet the resolution criterion shaded in [609]. By superimposing all the specific resolution maps of the various peak pairs a single figure, called an overlapping resolution map (ORM), is generated. Areas in the parameter space where the desired separation can be achieved for all solutes in the mixture are easily identified. In agreement with the results from Figure 4.28, the optimum region for the separation predicted by the overlapping resolution map (Figure 4.29) is the same. The overlapping

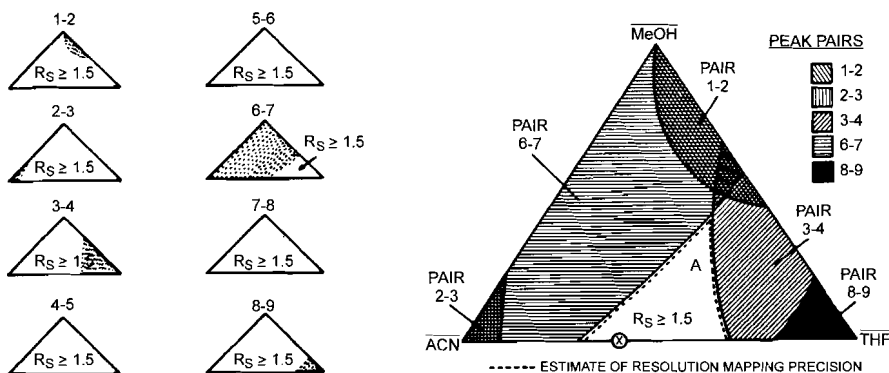


Figure 4.29. Peak-pair resolution maps and an overlapping resolution map for the separation of nine substituted naphthalene compounds by reversed-phase chromatography illustrated in Figure 4.28. (From ref. [603]; ©Elsevier)

resolution map does not define a unique solution but indicates a region or regions where successful separations can be obtained. Also, the overlapping resolution map does not explicitly use the separation time as a criterion for the quality of the separation but this is handled by the experimental design. The solvent strength throughout the parameter space is approximately constant, and therefore, the separation time for all chromatograms within the parameter space should also be approximately constant.

The overall scheme for iterative methods involves selection of the parameter space, data collection at model points, peak labeling, modeling of the retention surface, and calculation of response surfaces followed by the prediction of an optimum value [551,555,560,600,610]. The initial steps for selectivity optimization are similar to those discussed for the mixture-design approach. Linear or non-linear plots of the retention factor ($\ln k$) against mobile phase composition for each solute are constructed for the entire range of ternary mixtures derived from the pairs of binary pseudocomponents. A response surface is then constructed from the modeled retention surface using a suitable objective function. An estimate of the best mobile phase composition for the separation is made from the response surface. The sample is then chromatographed with this mobile phase composition and the quality of the chromatogram assessed. In the event that it is found to be inadequate the new retention data for each solute are then used with the original data to refine the response surface. The new plot is used to define a second predicted optimum composition and a separation conducted with this mobile phase. This process is repeated, as required, until sufficient iterations have been conducted to produce a model that is sufficiently close to the true behavior to enable an optimum separation to be predicted. The characteristic feature of an iterative regression design is that the response surface is derived indirectly from the retention surface of individual solutes, which are easier to model. The number of data points and the retention model are not fixed in advance but updated continuously by verifying the predicted optimum. The retention surfaces are described by a succession

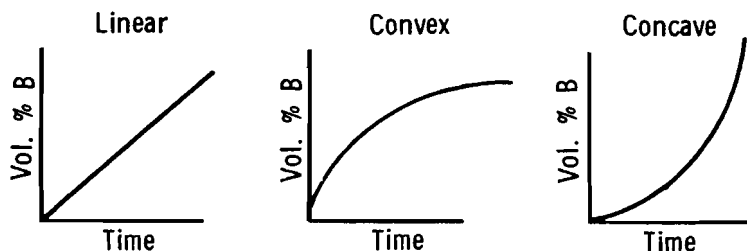


Figure 4.30. Idealized shapes for mobile phase gradients.

of linear or quadratic segments until the true curves have been found with sufficient accuracy. Among the advantages of the iterative approach are that an impression of the complete parameter space is maintained throughout the procedure and the number of chromatographic separations required is adapted to the degree of non-linearity of the retention surfaces. Among the disadvantages are that the procedure is computationally intensive, particularly if a large number of parameters have to be optimized. Since it relies on an objective function to determine the quality of the chromatogram, it suffers from all the problems associated with matching the selected function to the goals of the separation. Since it relies on accurate retention data for each solute in all chromatograms, peak tracking is required.

4.4.6 Optimization Strategies for Gradient Elution Separations

Isocratic and gradient elution methods are complementary separation techniques. Isocratic methods are generally preferred because of convenience, simplicity and reproducibility, but are inappropriate for samples containing components with a wide retention range (section 1.4.5). Gradient methods are also preferred for the reversed-phase separation of macromolecules, such as biopolymers, owing to the large variation in retention accompanying small changes in isocratic mobile phase composition. The characteristic feature of gradient elution is the programmed increase in mobile phase solvent strength during the separation [611]. Changes in solvent strength are accompanied by a simultaneous change in selectivity for many compounds. Thus, gradient elution provides an effective means of selectivity optimization for samples with a wide retention range in a reasonable separation time with sharper peaks for all sample components.

Most separation problems requiring gradient elution can be solved using binary solvent gradients in which the volume fraction of the strong solvent is progressively increased with time. Ternary and quaternary solvent gradients are useful for fine tuning selectivity but are more difficult to program and optimize [611-613]. Some typical gradient profiles are illustrated in Figure 4.30. Linear gradients are the most widely used because they are easier to program and can be describe by simple approximate theoretical relationships. Non-linear and segmented gradients are required for separations obtained by optimized linear gradients that contain a large number of overlapping peaks, or a small number of widely separated peaks, or consist of several regions with differ-

ent optimum selectivity. Non-linear gradients are also preferred for the reversed-phase separation of homologous or oligomeric mixtures, where resolution is observed to decrease with increasing retention (molecular weight) in a linear gradient [614]. A convex gradient provides a faster increase in the concentration of the stronger solvent at the beginning, and a slower increase towards the end, of the separation than a linear gradient. It reduces band spacing at the beginning and increases it towards the end of the chromatogram as well as shortening the total separation time. A concave gradient produces an opposite effect to a convex gradient with wider, better-resolved peaks at the start of the chromatogram and sharper, less-well-resolved peaks appearing at the end. Complex gradients are constructed by combining gradient segments with different shapes, isocratic segments and step increases in the stronger solvent. Complex gradients are useful for the efficient optimization of chromatograms with an uneven peak distribution, particularly those containing alternating regions that are relatively crowded and empty [611,615,616]. Since the gradient shape for earlier segments can affect the separation of peaks in a later segment, the design of an optimal gradient program containing several different segments can require a large number of trial-and-error experiments or accurate computer simulation to establish the desired experimental conditions. In essence, the more complex the gradient program, the more time that will be required for method development. Consequently, complex gradients should be reserved for difficult samples that failed to yield an acceptable result with a simple continuous gradient. In method development one may end up with a complex gradient for an optimized separation but a complex gradient should never be the starting point.

4.4.6.1 Linear Solvent Strength Gradients

The exact description of retention under gradient elution conditions is possible only by differential equations. In most cases these equations can be simplified to reasonable forms, of which the linear solvent strength model is simply one of several proposed models to predict separation properties under gradient elution conditions [611]. It is a relatively simple model that relates the gradient elution process to analogous isocratic separation conditions. It is generally sufficiently accurate to predict retention and resolution in gradient separations by computer simulations based on a small number of experiments [594,616-620]. The model is based on the co-linear relationships between the change in solvent strength during the gradient separation and the approximate effect of the strong solvent on retention in isocratic reversed-phase, normal-phase, ion-exchange and hydrophobic interaction chromatography. These relationships are approximate and are expected to be reliable for only a modest range of mobile phase compositions. The general retention equation for a linear solvent strength gradient, analogous to Eq. (4.3) for isocratic reversed-phase separations, is given by

$$\log k_i = \log k_o - b(t / t_M) \quad (4.37)$$

where k_i is the solute retention factor at the column inlet, k_o the isocratic retention factor in the mobile phase composition at the start of the gradient, b the gradient steepness

parameter, t the time after the start of the gradient and sample injection and t_M the column hold-up time. Eq. (4.37) provides a reasonable fit to experimental data, at least for the optimum retention factor range ($0.5 < k_i < 20$). The gradient steepness parameter should remain constant throughout the gradient program and hence have the same value for all sample components, a condition that is rarely exactly true for normal operating conditions. The gradient steepness parameter is defined by Eq. (4.38)

$$b = \Delta\phi S V_M / F t_G \quad (4.38)$$

where $\Delta\phi$ is the gradient range (change in the volume fraction of the stronger solvent from the start to the end of a binary solvent gradient), the S -value the average change in isocratic $\log k$ for unit change in the volume fraction of stronger solvent (section 4.3.1.1), t_G the gradient time (the time from the start to the end of the gradient), V_M the column hold-up volume and F the mobile phase flow rate. The gradient steepness parameter will vary with the S -value, which is only roughly constant for similar compounds. Systematic variations in the S -value are generally observed for homologs, oligomers and for compounds with a similar parent skeleton but different numbers of functional groups. Macromolecules also exhibit systematic variation of S -values as a function of molecular weight. For macromolecules the S -value can be 30- to 100-fold greater than that for small molecules. For these substances, retention is extremely sensitive to changes in mobile phase composition and typical gradients require different experimental conditions than those used for small molecules [616].

A change in the quantity $1/b$ or t_G in a linear solvent strength gradient has a similar effect on a separation as a change in the retention factor for isocratic separations. Thus, analogous to isocratic separations, it should be relatively straightforward to predict changes in peak width, resolution and separation time accompanying changes in the gradient steepness parameter in gradient elution chromatography, Table 4.18 [616-620]. The average or effective retention factor, k^* , provides the link between gradient and isocratic separations. In an ideal linear solvent strength gradient the effective retention factor is roughly equal for all components eluting at different times during the separation, all bands are eluted with approximately constant band widths, and the resolution between adjacent band pairs with similar values of the separation factor are equal. If the gradient range is shortened to minimize the separation time then the gradient time must be reduced proportionately to maintain a constant value for b (Eq. 4.38), otherwise the optimum band spacing will not be retained and resolution declines. Optimum values of b are generally in the range 0.43 to 0.09 corresponding to a k^* about 2-10. An increase in k^* , analogous to an increase in isocratic retention factors, causes resolution to increase initially then level off, bands to become broader and the separation time to increase. Increasing the flow rate with all other conditions the same results in a decrease in b and changes in the separation similar to those discussed for the gradient time and the gradient range. Note that the optimum gradient time for a given value of b depends on the column hold-up time and should be adjusted accordingly when columns of different dimensions are used.

Table 4.18

Relationships for chromatographic parameters in a linear solvent strength gradient

Symbols are identified in the text: t_D the dwell time for the chromatograph (the time it takes a change in mobile phase composition to pass from the gradient mixer to the column inlet) and k_Z the isocratic retention factor in the terminal solvent for the gradient

(i) Retention

(a) Sample strongly retained at the beginning of the gradient (large k_0)

$$t_g = (t_M / b) \log (2.31k_0b + 1) + t_M + t_D$$

(b) Sample weakly retained at the beginning of the gradient (small k_0) and t_D is significant

$$t_g = (t_M / b) \log [2.31k_0b(1 - x) + 1] + (1 - x)t_M + t_D$$

where $x = (t_D / t_M k_0)$

(c) Solute elutes after the gradient is complete

$$t_r = t_G + t_M + t_D + (1 - r)k_Z t_M$$

where $r = (10^8 / 2.31k_0b)$ and $g = (bt_G / t_M)$

(d) Average or effective retention factor k^* (retention factor for a band when it has eluted half way through the column) assuming k_0 is large

$$k^* = (1 / 1.5b)$$

$$k^* = 0.87t_{GF} / V_M \Delta \phi S$$

(e) Retention factor at the time the band elutes from the column k_f

$$k_f = (1 / [2.3b + (1 / k_0)])$$

(ii) Band width

$$W = 4.4t_M(1 + k_f)\sqrt{N}$$

(iii) Resolution

$$R_S = [\sqrt{N} / 4] [\alpha - 1] [k^* / (1 + k^*)]$$

(only true if the two bands have similar S-values)

The band width in gradient elution separations is the result of three more or less independent processes: the normal sample band broadening as the band moves through the column; the band compression phenomenon arising from the faster migration of the rear of the band in gradient elution compared to the equal migration of all parts of a band in isocratic elution; and to the instantaneous retention factor of the band, k_f , as it leaves the column. The plate number for a gradient separation corresponds to an average retention factor, k^* , and this is the value used for the resolution equation, Table 4.18. Optimum resolution in the gradient elution mode is expected for $0.5 < k^* < 20$ and is adjusted by varying the parameters controlling the gradient steepness factor, discussed above. An optimum value for b is set by choosing an average retention factor in the desired range, for example $k^* = 3$, and using this value to calculate b from the relationship between b and k^* . The value for b is then used to fix the experimental variables defined in Eq (4.38), such as the gradient time and range (t_G and $\Delta \phi$).

4.4.6.2 Method Development in Gradient Elution

The initial and final mobile phase compositions and the gradient shape are the properties required to define a suitable gradient for a separation. Solvent selection rules are the same as for isocratic separations (section 4.6.2). The initial mobile phase composition

influences the separation of the first few bands in the gradient chromatogram but is less important for the separation of later eluting bands. The initial mobile phase composition should be selected to provide optimum resolution for the early eluting bands in the chromatogram with minimum time delay before the appearance of the first peak. The terminal mobile phase composition influences the selectivity of the separation (relative peak positions) and the retention time and peak shape of late eluting peaks. When bands elute long after the end of the gradient program is complete a stronger terminal solvent is required. If the terminal solvent is too weak, the separation time may become inconveniently long and later eluting bands broadened and difficult to detect. Within these constraints, the gradient profile is selected and optimized to obtain an even distribution of bands throughout the chromatogram.

Systematic optimization of linear solvent strength gradients begins with a full-range gradient from 5 to 100% strong solvent with conditions selected to give values of $k^* \approx 2$ [588,615-618]. This gradient allows a decision to be made on whether an isocratic or gradient separation should be pursued. It allows an initial estimate of the mobile phase solvent strength for an isocratic separation (section 4.4.3) or gradient range ($\Delta\phi = \phi_z - \phi_o$) for a gradient separation. Given values for the experimental conditions (t_G , ϕ_o , ϕ_z , V_M , F and t_D) and the isocratic parameters S and k_W it is possible to predict values of solute gradient retention times from the relationships in Table 4.18. Values of S and k_W can be obtained from isocratic experiments but are more conveniently obtained from two gradient runs where only the gradient steepness parameter is varied (usually by choosing values of t_G that differ by a factor of 3 to 4). Solution of the simultaneous equations provides values for b and k_o from which S and k_W are obtained. Once these values are acquired for each solute computer simulation allows optimization of band spacing as a function of the gradient parameters. In addition, modest improvements in resolution are possible by varying the column conditions (e.g. length, particle size, flow rate, etc.), also evaluated by computer simulation. Inadequate separations at this point require a change in system selectivity. System selectivity is changed most effectively by varying the following parameters in the order: temperature (least effective) < column type < solvent type. Although temperature is the least effective parameter for changing selectivity the simultaneous optimization of temperature and solvent strength is a straightforward process using computer simulation [621-625]. Experimental retention times are required for two gradient runs with different gradient times at two temperatures. This allows S -values and $\log k_W$ to be determined as a function of temperature and then retention to be predicted for all gradient conditions and any temperature. Results are usually displayed as resolution maps (a display of the resolution for the least separated band pair with coordinates of temperature and gradient time). This allows selection of the optimum temperature and gradient conditions for the separation by simple inspection of the resolution map. When faced with failure at this point optimization can be started over again using a different strong solvent, a different stationary phase or more complex gradient shapes can be considered. Alternatively, the final chromatogram for the best separation can be divided into clusters containing unresolved peaks. The gradient conditions for each cluster are then optimized one at a time, starting from the

first cluster. This process is completed iteratively since the separation achieved in any segment is affected by all preceding segments. When failure results from an inadequate peak capacity an effective strategy is to divide the separation into a number of separate optimized gradient runs such that all relevant components are separated in one or the other of the runs [623].

The advantage of the simulation approach is that several changes to the gradient conditions can be made and evaluated without the need for additional experimental data. On the other hand the predictions obtained depend on the quality of the model employed. For the linear solvent strength model the results are generally good in spite of a number of questionable approximations in its formulation. Alternative strategies for optimizing gradient conditions include simplex optimization and the mixture-design statistical approach [600,626]. These approaches are similar to those used to optimize mobile phase composition for isocratic liquid chromatography and retain the same strengths and weaknesses (section 4.4.5). A general structure-driven model for retention in gradient elution reversed-phase chromatography based on the solvation parameter model (section 4.3.1.3) has been proposed but so far has not been used as a method development tool [278,627].

4.4.7 Automated and Expert Systems for Method Development

In spite of considerable progress in developing automated systems for method development in liquid chromatography the final objective of a “smart box” that can solve a wide range of problems remains an elusive goal [375,550-552]. There are several stumbling blocks. The selection of the separation model and the parameter space is largely a matter of judgement, but has a profound impact on the success of the optimization step. The objective function remains a weak link of computer-aided optimization procedures, since the quality of a chromatogram is not easily encoded by a single numerical value (section 1.6.2). The objective function is not unique and several different chromatograms can produce the same value, even though some separations would be judged better than others. Few laboratories have a wide range of software to choose from; many of the procedures discussed in earlier sections require software restricted to a particular instrument or is not commercially available. When the time required for trial separations, column re-equilibration and peak tracking are considered, these approaches are not particularly rapid and an experienced chromatographer might well develop an acceptable separation in a shorter time. The most useful, and therefore used software products, are those which assist the chromatographer in the decision making steps by performing calculations for further experiments or simulate the effect of further experiments. In other words they provide advice but do not frame the questions or dictate what is done with that advice. For the experienced chromatographer they are time saving tools, but for the novice they can be a shortcut to nowhere, and sometimes, not even a shortcut.

Since experienced chromatographers are neither omnipresent nor always agreeable to providing guidance at the time it is required, attempts have been made to make

their knowledge more accessible in the form of computer-based artificial intelligence systems, generally referred to as expert systems [628-631]. An expert system is a computer program that represents and reasons with knowledge of some specialist subject with a view to solving problems or giving advice. Such a program is created by acquiring the expert's knowledge, encoding that knowledge in a computer program language and making that knowledge accessible through a user interface. The knowledge is more detailed than calculation algorithms and the user interface has to be more flexible than required for typical computer programs operating on numerical input. Expert systems contain a considerable amount of "word information" and it is the challenge of the computer engineer to organize this information in logical sequences and make it available to the user at an appropriate level, quality and quantity. Those expert systems currently available are largely designed for scientists with some experience of method development. Inexperienced chromatographers find them difficult to use, have difficulty in framing the right questions or providing the correct information, or accept their advice implicitly and are sometimes disappointed and unsure what to do next. Expert systems can plan the whole or part of (usually) a reversed-phase separation, predict the values for experimental data, diagnose problems or identify sample components. The literature suggests that the most successful expert systems are those that tackle a single aspect of the method development process rather than integrated systems which attempt to tackle the whole process. General use of expert systems for method development is not common at present and their evolution went into a stall phase during the last decade. A successful application of expert systems is the development of troubleshooting programs to diagnose instrument component failure or inform the user of corrective actions.

4.5 COLUMN PREPARATION

Few laboratories have developed their own facilities for packing conventional and small-bore columns, preferring to rely on commercial manufacturers for their needs. This is understandable since packed columns containing any common phase can be obtained at a reasonable cost and with a guarantee of acceptable performance. Column packing generally requires equipment not found in all laboratories. It is not an exact science and requires some time to acquire the practical skills needed for proficiency. Similar packing materials often require different packing procedure to produce columns of acceptable performance. The general literature contains many conflicting statements and generalizations that are not adequately tested. Although good columns can be prepared using a variety of different procedures, attempts to optimize the many variables and identify the best methods have met with limited success [632,633]. Except in those laboratories involved in the design of novel stationary phases the knowledge of how to pack good columns has now been lost. Recent advances in column packing techniques have largely occurred in the commercial sector and this knowledge is treated as proprietary information.

4.5.1 Column Blank

The column blank must be able to withstand the pressure used for column packing and normal operation and be chemically resistant to a wide range of mobile phases [23]. For the majority of applications seamless and polished 316 stainless steel meets the above requirements and is the preferred material. It is mechanically strong, available in a wide range of dimensions, and is inert to most solvents. Exceptions are aqueous solutions of halide salts, particularly at low pH, and some organic solvents, which can induce corrosion upon prolonged contact with the metal [634,635]. Alternatives to stainless steel include heavy-walled glass tubing, glass or polymer lined stainless steel tubing and plastic tubing. Heavy-walled glass tubing is used for cartridge columns and has many desirable features except for high-pressure stability. Columns with internal diameters of 3.0 mm are usually rated as safe to about 200 atmospheres. PEEK, poly(ether ether ketone) is used to prepare columns of various diameters down to about 0.5 mm, largely for applications where a metal-free system is important (e.g. some applications in biochromatography and ion chromatography). Plastic columns with pressure-deformable walls inserted in a hydraulic radial compression module are used mainly for preparative chromatography [636]. Fused-silica capillary columns, similar to those used in gas chromatography, with diameters up to about 0.5 mm have virtually replaced all other materials for the preparation of packed capillary columns. These columns are strong, flexible and optically transparent, enabling on-column detection techniques to be used.

Prior to packing, the interior wall of the empty stainless steel column must be cleaned and polished. It is particularly important that the column blank has a smooth inner surface and that residual grease and metal fines from the tube drawing process are removed. This is achieved by a thorough washing sequence involving aqueous and organic solvents. Dilute acids and detergent solutions may also be included in the washing sequence. The air-dried tube is then polished internally by passing a lint-free cloth attached to a nylon thread or a pipe cleaner through the column several times [637]. The inner wall of the polished column should be smooth and reflective when viewed against the light, and free from any indentations and/or burrs.

The packing material is retained and protected from the intrusion of particle matter by sintered metal or porous polymer frits or screens. These may be either inserted into the column ends or made an integral part of the column end fittings. Their average pore diameter should be less than the particle size of the column packing (typically 0.5 or 2 μm). The most common column end fittings are stainless steel internal fittings with female threads in the fitting body and a male nut. This arrangement provides a more durable connection when fittings are frequently reused. Column end fittings are not standardized, and although different fittings may look alike, they may have different thread sizes, which makes them non-interchangeable. Finger-tightened fittings, which rely on a polymeric ferrule to make the seal between the tubing and the fitting body, are becoming increasingly popular. Finger-tightened fittings are used with cartridge columns consisting of the tubular column with the packing material retained by frits pushed into the end of the tube and a cartridge holder that connects the column

to the instrument with a reusable compression fitting or compression device. The cartridge format provides a no tools approach to column installation and allows the same connection hardware to be reused each time a column is exchanged. Other connections are usually made with zero-dead volume unions and short lengths of stainless steel capillary tubing. For metal-free systems, fittings, unions and capillary connecting tubing made from pressure resistant polymers such as PEEK are employed.

4.5.2 Column Packing Methods

The choice of column packing method depends on the mechanical strength of the packing, its particle size and surface properties, and on the column dimensions. Rigid particles with an average diameter greater than 20 μm can be dry packed, usually without much difficulty. Particles of smaller diameter have a high surface energy to mass ratio and when dry packed result in an unstable packing structure with many voids, unsuitable for liquid chromatography. High-pressure, slurry-packing techniques are used to pack small diameter particles.

The critical steps in slurry packing are the preparation of the slurry, the filtration of the slurry through the column to form the particle bed and the compaction and conditioning of the particle bed. The ease of preparing columns with acceptable performance declines with decreasing particle size, decreasing column diameter and increasing column length, largely independent of the packing technique. The particles have to enter the column at high velocity in order to overcome frictional forces from viscous flow around the particles and at the wall. The packing speed is dependent on the applied pressure, the slurry viscosity and the particle size. The kinetic energy with which a particle hits the accumulating bed depends on its mass (particle size) and velocity, as well as the particle shape and liquid viscosity. At sufficiently high packing pressure the choice of packing method and slurry liquid is less critical for large particles (e.g. 10 μm) with optimum packing conditions achieved over a reasonable range of experimental parameters. Reducing the particle size lowers the kinetic energy of the particles, which must be compensated for by increasing the particle velocity. Higher pressures and lower viscosity slurry liquids will be more favorable for packing smaller diameter particles (2-5 μm) [632]. Most columns have been packed at a constant pressure using high-pressure gas amplification pumps. To promote a homogeneous packing structure both pressure programmed [638] and constant velocity packing have been advocated [639]. For columns of conventional diameters constant velocity packing is limited by the availability of pumps capable of maintaining the optimum packing flow rate at high pressures. Conventional analytical columns are generally packed using the constant pressure packing technique.

The main requirements for the slurry packing liquid are that it must thoroughly wet the packing, provide adequate dispersion of the packing material, and be easily washed out of the column after packing [633,640-642]. Since packings with different surface properties and densities are used in liquid chromatography, a wide range of common solvents and solvent mixtures have been used for this purpose. Balanced-density slurry

techniques are commonly used for packing particles with diameters of about 10 μm and to a lesser extent for packing small bore columns with smaller particles [637,643,644]. Balanced-density slurry liquids are usually mixtures of various proportions of a high-density liquid (e.g. carbon tetrabromide, carbon tetrachloride, etc.) and a low-density liquid (e.g. methanol) that is adjusted to a similar density to the packing material. Segregation by sedimentation is slow in liquids of similar density to the suspended particles. The optimum slurry liquid combination is easily established by trial and error. After sonication the balanced-density slurry should be stable at room temperature for at least 30 minutes without signs of sedimentation. Particle sedimentation towards the bottom of the flask indicates that the concentration of the denser slurry liquid should be increased. The opposite is true when sedimentation occurs against the influence of gravity. Settling out of the suspension can also be avoided by using slurry liquids of high viscosity, such as mixtures of isopropanol and glycerol [645]. These liquids, however, increase the resistance to flow through the column, so that the packing procedure is slow or requires a higher packing pressure than other methods. Particles with diameters less than about 7 μm sediment slowly at room temperature in liquids with good dispersion properties, such as chloroform, acetone, methyl *tert*-butyl ether and methanol [633,646,647]. These solvents are suitable for packing columns with small diameter particles if the packing procedure is rapid.

Capillary columns with internal diameters from 0.04 to 0.5 mm are packed using the slurry packing method [648-650] or by displacement using supercritical fluid carbon dioxide [651-654]. A high slurry concentration in a low viscosity liquid and a stepped increase in packing pressure over time are generally used in the slurry packing method. Aqueous slurries containing a surfactant were also shown to be effective [650]. Housing the column in an ultrasonic bath during the packing and consolidation process increases the homogeneity of the packed bed. For the carbon dioxide method dry packing material is placed in the packing reservoir and displaced into the column by liquid or supercritical fluid carbon dioxide. In a typical arrangement the column is housed in an ultrasonic bath containing water at 40-70°C and a linear restrictor is attached to the exit end of the column. During transit from the packing reservoir to the column exit the packing solvent is converted from the liquid to the supercritical state. The supercritical fluid carbon dioxide flowing through the heated column section has a faster velocity and a lower viscosity than in the liquid state, which accelerates the packing process and improves the formation of a homogeneous bed. The principal advantage of the supercritical fluid packing method is that it allows longer columns with smaller diameter particles to be prepared. Columns in a single length up to 10 m can be prepared, although in practice shorter columns (< 1 m) are generally used in liquid chromatography. Longer columns are suitable for supercritical fluid chromatography.

Both packing methods result in packed capillary columns that are more permeable than conventional diameter columns. The number of particles in contact with the wall increases proportionally with a reduction in the column internal diameter exerting a stabilizing influence on the packed bed to flow and pressure variations in spite of their lower packing density. The narrow internal column diameter and packing

density conspire to provide favorable kinetic efficiency under conditions that would be disastrous for conventional diameter columns [648,649,653].

In recent years some longstanding views of the column packing process have been challenged based on a combination of experiment and theories accepted in colloid chemistry and rheology. A common thread in many early studies was the belief that optimum column performance was associated with obtaining a maximum packing density. It is now established, however, that the maximum column efficiency for slurry packed columns is associated with an optimum packing density, which is somewhat lower than the highest packing density that can be obtained [653,655]. Low packing densities are undesirable because the column bed is prone to collapse but within the range of stable bed structures higher than optimum packing densities are undesirable because they lead to an increase in the radial flow heterogeneity of the column resulting in lower separation efficiency. Based on early studies of dry-packed columns it was accepted that the packing density close to the column wall was lower than at the column core due to enhanced accumulation of coarse particles at the wall promoting radial flow heterogeneity. For slurry packed columns wall friction ensures that the packing density is highest at the wall and lower at the core [655]. The friction between the wall and the column packing is responsible for supporting the packing structure. These forces are reasonably elastic so that when the pressure confining the packed bed is released the column bed can relax to its original length [647]. The friction between the bed and the wall is expected to slow, delay and dampen the bed movement. The influence of wall friction on the radial packing density will depend on the column diameter and will be most intense for narrow diameter columns. The flow profile of the packing slurry into the column blank is parabolic resulting in a heterogeneous radial particle distribution. Compression from particles above and the shear stress from the flow of solvent through the immature bed result in movement of particles across the column diameter from regions of high to low packing density [653,655,656]. This results in an increase in the radial packing homogeneity. For narrow diameter columns this process is assisted by sonication [650,653] but the influence of sonication seems largely muted for conventional diameter columns [647]. Although generally accepted that the slurry liquid should have favorable dispersion properties for the particles it has been suggested that the pushing or packing liquid used to stabilize and condition the initial packed bed should be a coagulating liquid [642]. In general there is little agreement as to whether the chemical properties of the slurry liquid and the initial packing of the column or the properties of the packing liquid and the consolidation of the bed is the key step in the column preparation procedure.

4.5.2.1 Dry-Packing Procedures

Rigid particles with diameters greater than 20 μm can be dry-packed efficiently by the tap-fill method using relatively simple apparatus. The empty column is held vertically with an end fitting at its lower end and a small amount of packing material, equivalent to about 3-5 mm of column bed, is added from a funnel. The packing is consolidated by tapping rapidly and firmly on a hard surface while lightly rapping on the side

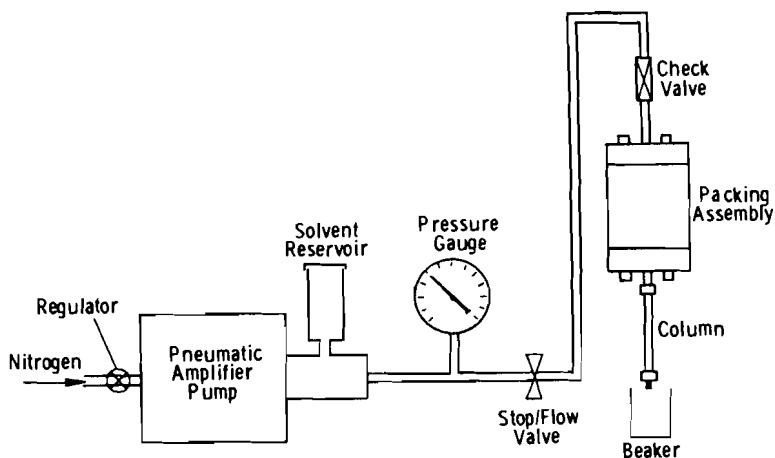


Figure 4.31. Down-fill slurry packing apparatus. (From ref. [658]. ©Elsevier)

of the column at the approximate packing level and rotating the column slowly. Some authors state that vertical tapping during the packing process is detrimental to column performance and recommend only lateral tapping at the level of the column packing [657]. Further increments of packing are added and the process is repeated until the column is filled, further consolidated, and then the inlet fitting attached to the column without disturbing the column packing. The packed column is then attached to the liquid chromatograph and equilibrated with the mobile phase until no further bubbles appear at the column outlet and the column pressure and flow rate have stabilized.

It should be noted that incremental addition of the column packing, followed by consolidation of the packed bed, generally gives better results than bulk filling. Also, lateral tapping should be performed with a hard metal tool, vibration devices similar to those used to pack gas chromatography columns should not be used, since they tend to produce particle segregation across the column diameter. This results in a heterogeneous bed with large particles near the wall and the smaller particles at the center.

4.5.2.2 Down-Fill Slurry Packing

Slurry packing techniques are required for the preparation of stable columns with rigid particles of less than 20 μm in diameter. The same general packing apparatus, Figure 4.31, can be used to pack columns by the balanced-density slurry, liquid slurry, or viscous slurry techniques [658]. Down-fill slurry packing is the method of choice for small bore columns and packed capillary columns.

Typical slurry concentrations are 1-30% (w/v) and have to be optimized for each liquid and packing combination. Before packing, the slurry should be thoroughly degassed and dispersed using an ultrasonic bath. The size of the packing reservoir should be scaled in proportion to the dimensions of the column blank and should not be overly large. Stainless steel bombs with finger-tightened connections are commercially

available for packing conventional and semipreparative columns; for narrow bore columns sections of stainless steel tubing will serve as a slurry reservoir. Some reservoirs have a conical internal shape to promote a smooth passage of the slurry into the column [643].

Using Figure 4.31 as a guide, the down-fill slurry column packing procedure will be described [637,639,643,645,658]. A retaining porous frit or screen and standard end fitting are attached to the lower end of a clean column blank. The top end of the column is connected to a short length (3-5 cm) of connector tubing, which in turn is attached to the base of the slurry reservoir. The purpose of the connector tube is to serve as a mini-reservoir; it guides the slurry into the column blank and ensures that the column is completely filled when the packing procedure is complete. It is important that the internal diameter of the connector tube and the outlet fitting of the slurry reservoir are the same as that of the column blank. This will ensure a rapid and smooth delivery of the slurry into the column without subjecting the packing to undue sheer forces.

The column blank and connector tube are filled with either the slurry liquid or a solvent of higher density. The side of the column is tapped with a metal rod to ensure that air bubbles are not left in the column during this step. The liquid is retained in the column by attaching either a stopcock or a terminator end fitting to the bottom of the column. The stop-flow valve is then opened to fill the reservoir and connector tube with the packing liquid. With the stop-flow valve closed the packing pressure is then established (5,000-60,000 p.s.i.) At this point the degassed slurry is rapidly added to the slurry reservoir which is then closed. Any air pockets are displaced with additional slurry solvent. The reservoir is then immediately attached to the pump line, the stopcock opened or the terminator removed, and the stop-flow valve opened. The packing process is rapid for conventional diameter columns (except for the viscous slurry method); the actual filling of the column is usually completed within a few seconds. However, a constant pressure is maintained until about 20 column volumes of packing liquid have been pumped through the column. The stop-flow valve is then closed and the pressure allowed to bleed through the column until the liquid flow ceases. The column performance and stability is often improved if the freshly packed column is further consolidated without removing it from the packing assembly. The consolidation process is extended until the column flow rate is stable. The column is then removed from the packing apparatus and excess packing material removed from the top of the column with a razor blade. Afterwards a screen or frit and end fitting are attached to the column detector end and the column is ready for testing and use.

4.5.2.3 Up-Fill Slurry Packing

The up-fill slurry packing method is used for packing conventional diameter columns. It is unsuitable for packing small diameter columns. It is easily adapted to the simultaneous packing of several columns and generally used with low viscosity slurry liquids. The principle of the packing method is based on the upward displacement of a stable slurry into the column blank from a reservoir whose contents are continuously diluted by incoming pressurized solvent [646,659]. Dilute slurries (1-10% w/v) and

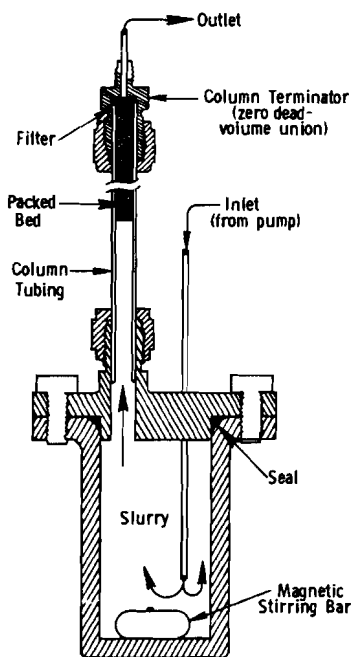


Figure 4.32. Up-fill slurry packing apparatus.

continuous stirring are used to minimize agglomeration of the packing slurry in the reservoir. This is important for particles larger than $5\text{ }\mu\text{m}$ in diameter to ensure that the particles remain suspended during the up-flow packing process. The viscosity of the slurry liquid determines the time required to pack the column and the impact velocity of the particles during the packing process. The controlling factor for packing columns by the up-fill method is that the velocity of both the particles striking the bed and the liquid transporting them must be high enough to prevent the particles from falling back into the reservoir. Yet the required velocities are still relatively slow. As a result, the up-fill method allows the greatest opportunity for ordering of the bed to produce a densely packed structure.

To pack a column, the slurry is poured into the reservoir, Figure 4.32. It is stirred continuously to maintain a homogeneous suspension. With the lid of the reservoir securely in place and the outlet valve open, the slurry liquid is pumped gently into the attached column until the first drop of slurry appears at the end of the column. This purges trapped air from the system. The outlet valve is then closed and the packing pressure established (2,000-7,500 p.s.i.) The valve is then reopened, and the slurry is forced into the column. The packing process is complete when the flow rate of solvent through the column has stabilized. The pump and magnetic stirrer are stopped, the pressure is allowed to bleed away, and the column is disconnected from the reservoir.

An end fitting and frit are attached to the column inlet end and the column is ready for testing and use.

4.5.2.4 Packing Semirigid Particles

Packing procedures for semirigid particles are similar to those used for rigid particles, except that the column packing pressure is limited by the lower mechanical strength of the packing. Polymeric ion-exchange resins are usually packed by the salt-balanced-density slurry method. Porous polymers must be swollen in the slurry solvent prior to packing. The polymer is packed into the column at a pressure dependent on the mechanical strength of the swollen polymer beads, usually less than 5,000 p.s.i. Semirigid polymer gels are usually packed by the balanced-density slurry packing technique after first being swollen for several hours in the slurry liquid. These gels are normally packed at pressures less than 3,000 p.s.i. These gels have a low density compared to silica and require lower density liquids for slurry preparation.

4.5.3 Evaluation of Column Quality

Most newly purchased columns are supplied with a test chromatogram together with information for a range of physicochemical properties determined for the batch of packing material supplied in the column. This typically includes descriptive material, such as the stationary phase and substrate type, whether endcapped or not; physical properties, such as the average particle size and the characteristics of the particle size distribution, the average pore size and characteristics of the pore size distribution, the pore volume, the specific surface area, the concentration of significant metal impurities, etc.; and chemical properties, such as carbon loading, concentration of bonded phase, etc. As useful as this information is, it is insufficient to establish how a column will behave for the separation of different sample types and to adequately compare the properties of different columns. More specific chromatographic tests are required for this purpose.

A new column, whether purchased or packed in the laboratory, should be tested before use, and periodically re-tested while in use. In this way the analyst can be sure that the column meets reasonable specifications for general performance and has a reliable method to monitor changes in column properties as a function of time or the type of samples analyzed. Routine column tests are simple, they require the generation of a single isocratic or sometimes a gradient elution chromatogram, yet reveal valuable information about the quality of the column packing and the success of the column packing process [660-666]. Other, more specific tests for reversed-phase packings are available to assess particular chromatographic properties, such as hydrophobicity, silanophilicity, complexation with metal impurities, ion-exchange properties and effective pore size distribution. These tests are non-destructive and can be supplemented by destructive tests to determine column stability as a function of mobile phase properties, e.g. pH (section 4.2.2.1).

Table 4.19

Test mixtures for routine quality evaluation of normal- and reversed-phase columns

<i>Normal-phase columns</i>	
(1)	Mixture: toluene, nitrobenzene and p-nitroaniline Mobile phase: isooctane-ethanol-water (84.5:15:0.5 v/v)
(2)	Mixture: naphthalene, m-dinitrobenzene and o-nitroaniline Mobile phase: hexane-dichloromethane-2-propanol (89.5:10:0.5 v/v)
(3)	Mixture: toluene, phenanthrene and nitrobenzene Mobile phase: hexane-acetonitrile (99:1 v/v)
(4)	Mixture: toluene, nitrobenzene, acetophenone, 2,6-dinitrotoluene and 1,3,5-trinitrobenzene Mobile phase: hexane-methanol (99.5:0.5 v/v)
<i>Reversed-phase columns</i>	
(1)	Mixture: resorcinol, acetophenone, naphthalene and anthracene Mobile phase: acetonitrile-water (55:45 v/v)
(2)	Mixture: uracil, phenol, benzaldehyde, N,N-dimethyl-3-toluamide, toluene and ethylbenzene Mobile phase: acetonitrile-water (65:35 v/v)
(3)	Mixture: acetone, acetophenone, anisole, benzene and toluene Mobile phase: acetonitrile-water (60:40 v/v)
(4)	Mixture: thiourea, phenol, 1-chloro-4-nitrobenzene, toluene, ethylbenzene, n-butylbenzene Mobile phase: methanol-water (80:20 v/v)
(5)	Mixture: uracil, toluene, acenaphthene, propylparaben, dipropylphthalate Mobile phase: methanol-water (65:35 v/v)

4.5.3.1 Routine Column Quality Evaluation

No single method exists for evaluating column quality. Test methods have to accommodate different stationary phase types and even a range of properties for the same stationary phase type. A suitable routine test procedure is easily designed by limiting the scope of the test to the determination of a few general column kinetic and retention characteristics. The solutes should be of low molecular weight to ensure favorable diffusion properties and of sufficient number and character so that one solute indicates the column hold-up volume ($k \approx 0$) and at least two components are adequately retained ($k \approx 2-10$) for an accurate assessment of retention and column efficiency. The solutes should have favorable detection properties (e.g. UV absorption) and the test sample (volume, sample solvent and amount) should not result in additional band broadening or overload the stationary phase. For reversed-phase columns of conventional diameters this is usually assured using mixtures of aromatic solutes at a concentration of 0.01 to 1.0 mg / ml each, dissolved in the mobile phase or a weaker solvent, with an injection volume of 5-10 μ l. The mobile phase should have a simple composition, low viscosity and preferably allow the test to be run under isocratic conditions. Some typical test mixtures for evaluating the quality of normal and reversed-phase columns are summarized in Table 4.19.

Having chosen the test mixture and mobile phase composition, the chromatogram is run producing results similar to Figure 4.33. The parameters usually calculated from the chromatogram are the retention factor of each component, the plate number for the unretained peak and at least one of the retained peaks, the asymmetry factor for each peak and the separation factor for at least one pair of solutes. The pressure drop for the

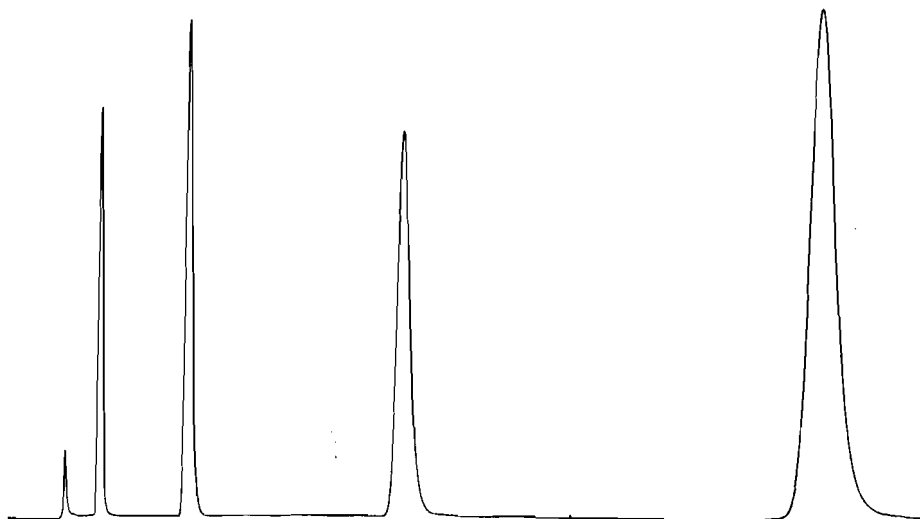


Figure 4.33. Typical routine column quality test chromatogram for a 30 cm x 4.6 mm column packed with an octadecylsiloxane-bonded silica packing of 10 μ m particle diameter. Test mixture: resorcinol (0.55 mg / ml), acetophenone (0.025 mg / ml), naphthalene (0.20 mg/ml) and anthracene (0.01 mg / ml) in acetonitrile, 10 μ l injected. Isocratic separation at 23°C with acetonitrile-water (55:45) as the mobile phase with a flow rate of 1.5 ml / min.

Solute	Retention factor	Asymmetry factor	N / m	Separation factor
Resorcinol	0.2	1.00	10,725	
Acetophenone	1.4	1.08	15,900	
Naphthalene	4.3	1.20	17,875	3.30
Anthracene	9.8	1.40	18,380	2.34

column at the optimum column flow rate should also be noted. The asymmetry factor (section 1.5.6), measured at 10% of the peak height in this discussion, is scrutinized first. A high asymmetry factor, especially for unretained or weakly retained peaks ($k < 3$), is typical of poorly packed columns (if instrumental contributions can be excluded). If only the unretained peak ($k < 1$) is asymmetric and/or there is a significant difference ($> 15\%$) between the plate counts for the unretained and retained peaks, then extracolumn effects are implicated and should be investigated prior to repeating the test. As a general guide, columns yielding asymmetry factors greater than 1.2 for solutes lacking strong specific stationary phase interactions are of poor quality and those with values greater than 1.6 are unacceptable.

The column plate count is scrutinized next. The plate count, by convention normalized per meter of column length, is expected to correlate primarily with the average particle size of the column packing, Table 4.20. These values are approximate upper limit values and depend on the experimental conditions and solute properties. Plate counts exceeding about 75 % of the tabulated values for well-retained solutes

Table 4.20

Theoretical column plate count and relative operating pressure at the optimum mobile phase velocity for conventional liquid chromatography columns

Column length (cm)	Average particle diameter (μm)	Column plate count	N / m	Relative operating pressure
25	10	12,500	50,000	1.0
25	5	25,000	100,000	4.0
15	5	15,000	100,000	2.4
5	5	5,000	100,000	0.8
10	3	16,700	167,000	4.4
5	3	8,300	167,000	2.2
3	3	5,000	167,000	1.3
3	2	7,500	250,000	3.0

are considered acceptable while columns with a plate height greater than 4 particle diameters are of poor quality. If the test solutes have acceptable asymmetry factors with lower than expected plate counts for well-retained solutes ($k > 3$) the column is probably poorly packed.

Under the test conditions, significant changes in the retention factors and separation factors for the test solutes compared to those found previously for similar columns indicate a change in the chemical properties of the packing material. The column might be well packed but the packing material has altered properties and is unlikely to provide separations similar to the previous column. The fault here lies in inadequate quality control of the synthesis of the column packing.

Significant changes in the column pressure drop under the same test conditions for columns of comparable length and particle size, indicates that either the column or its fittings are partially blocked. If cleaning the retaining frits and connecting tubing does not restore the pressure drop to the normal range then the column should be discarded. The results indicate that the packing probably contains an unacceptable amount of particles below average size restricting flow through the column.

4.5.3.2 Specific Column Quality Tests for Chemically Bonded Phases

The most common type of chemically bonded phases are prepared by reacting surface silanol groups of a porous silica substrate with a reactive organosilane reagent to attach the organosilane to the silica surface through siloxane bonds (section 4.2.2). Differences in reagents, reaction conditions and changes in the properties of the silica substrate are known to result in differences in the chromatographic properties of column packings obtained from the same or different manufacturers. These differences are manifested in changes in retention and selectivity generally caused by variation in the hydrophobicity, silanophilicity, ion-exchange capacity and activity induced by trace metal impurities. More specific quality evaluation tests are required to fully characterize changes in the above properties than is provided by the routine quality evaluation test (section 4.5.3.1). No single standardized test procedure has been developed but several test methods have been widely used, Table 4.21 [660-673]. Individual tests do not necessarily agree with

Table 4.21

Mixtures for specific property tests of reversed-phase columns

 α = separation factor, k = retention factor and A_s = asymmetry factor

Property	Measurement
(1) Walters [660]	
Hydrophobicity	α (anthracene / benzene) with acetonitrile-water (65:35)
Silanophilicity	α (N,N-diethyltoluamide / anthracene) with acetonitrile k (nitrobenzene) with n-heptane
(2) Kimata et al [662]	
Hydrophobicity	k for n-pentylbenzene with methanol-water (80:20) α (n-pentylbenzene / n-butylbenzene) with methanol-water (80:20)
Shape selectivity	α (triphenylene / o-terphenyl) with methanol-water (80:20)
Hydrogen-bonding	α (caffeine / phenol) with methanol-water (30:70)
Ion exchange (pH >7)	α (benzylamine / phenol) with methanol-0.02M phosphate buffer pH 7.6 (30:70)
Ion exchange (pH <3)	α (benzylamine / phenol) with methanol-0.02M phosphate buffer pH 2.7 (30:70)
(3) Sander and Wise [75,76,669]	
Shape selectivity	α (1,2,3,4,5,6,7,8-tetrabenzonaphthalene / benzo[a]pyrene) with acetonitrile-water (85:15)
(4) Engelhardt and Lobert [670]	
Metal impurities	100 A_s (2,2'-bipyridyl) / A_s (4,4'-bipyridyl) with methanol-water (49:51)
(5) Cruz et al [664]	
Metal impurities	Base peak efficiency (2,7-dihydroxynaphthalene) / base peak efficiency (2,3-dihydroxynaphthalene) with acetonitrile-25 mM ammonium acetate buffer pH 7.2 (25:75). 2,3-Dihydroxynaphthalene (300 mg / l) and 2,7-dihydroxynaphthalene (150 mg / l).

Comprehensive test mixtures

Neue and co-workers [665]

Uracil (16 mg / l), toluene (300 μ l / l) or naphthalene (60 mg / l), acenaphthene (200 mg / l), propylparaben or butylparaben (20 mg / l), dipropylphthalate (300 mg / l) or dibutylphthalate (400 mg / l), propranolol (400 mg / l) and amitriptyline (100 mg / l) or doxepin (100 mg / l) with methanol-20 mM phosphate buffer pH 7 (65:35 v/v).

Engelhardt and co-workers [671,672]

Thiourea (12 mg / l), toluene (870 mg / l), ethylbenzene (867 mg / l), ethylbenzoate (523 mg / l), aniline (81.7 mg / l), o-touidine (79.8 mg / l), p-toluidine (20 mg / l), N,N-dimethylaniline (38.2 mg / l), phenol (120 mg / l) with methanol-water (55:45)

one another since terms like hydrophobicity and silanophilicity, although reasonably well understood philosophically, are not so convincingly transposed into chemical tests.

The hydrophobicity of a stationary phase is generally characterized by the retention factor for a neutral compound, such as an alkylbenzene, or as the methylene group selectivity [266,664,666,667,671,674]. The latter is determined as the separation factor for adjacent members of a homologous series that differ only by a methylene group. Hydrophobicity scales are generally strongly correlated with the bonding density and chain length of the ligand, the ligand type and whether the phase is endcapped or not. It is usually only superficially correlated with the carbon loading. The hydrophobicity

Table 4.22

Variation of specific quality test values for reversed-phase sorbents

Solutes: PB = pentylbenzene, T = triphenylene; O = o-terphenyl; C = caffeine; P = phenol; and A = aniline (experimental conditions given in Table 4.21)

Stationary phase	Hydrophobicity		Shape selectivity $\alpha_{T/O}$	Silanophilicity		
	k_{PB}	$\alpha(CH_2)$		$\alpha_{C/P}$	$\alpha_{A/P}$ pH 7.6	$\alpha_{A/P}$ pH 2.7
Discovery C18	3.32	1.48	1.51	0.39	0.28	0.10
Hypersil 100 C18	7.66	1.53	1.40	0.42	1.01	0.25
Hypersil Elite	4.76	1.49	1.52	0.37	0.30	0.14
Hypersil ODS	4.44	1.45	1.28	0.38	1.04	0.64
Inertsil ODS	6.31	1.47	1.57	0.36	0.53	0.01
Kromsil C18	7.01	1.48	1.53	0.40	0.31	0.11
Lichrosphere RP18	7.92	1.48	1.73	0.54	1.39	0.19
Spherisorb ODS1	1.78	1.47	1.64	1.57	2.84	2.55
Spherisorb ODS2	3.00	1.51	1.56	0.59	0.76	0.23
Spherisorb ODSB	5.09	1.46	1.78	0.80	3.56	0.06
Symmetry C18	6.51	1.46	1.49	0.41	0.68	0.01
Zorbax Rx C18	5.68	1.57	1.61	0.54	0.55	0.11
Zorbax SB-C18	6.00	1.49	1.20	0.65	1.46	0.13
Zorbax SB-C8	1.97	1.37	1.08	1.27	0.81	0.12
Zorbax SB-CN	0.36	1.18	2.12	1.14	1.62	0.10
Zorbax SB-Phenyl	1.09	1.30	1.18	3.69	1.08	0.13

for different batches of the same column packing material is expected to show only a small variation [663,665]. For different column packings the variation in hydrophobicity is large, Table 4.22 [664]. This is not surprising given the wide range of bonding chemistries, reagents and substrates used in the production of chemically bonded phases for different applications. Although a number of different compounds are used to determine hydrophobicity there is fair agreement between the different test methods [666,667]. The retention factor for individual test solutes is generally a more discerning measure of hydrophobicity differences than methylene group selectivity, Table 4.22.

Sander and Wise have proposed a test method to determine the bonding chemistry used to prepare octadecylsiloxane-bonded silica packings based on the relative retention of three polycyclic aromatic hydrocarbons, benzo[a]pyrene (BaP), phenanthrophenanthrene (PhPh), and 1,2:3,4:5,6:7,8-tetrabenzonaphthalene (TBN) eluted with the mobile phase acetonitrile-water (85:15) [75,76,669]. On monomeric bonded phases the test solutes elute in the order $BaP \leq PhPh < TBN$ and on polymeric bonded phases $PhPh < TBN < BaP$. For monomeric phases with a high bonding density or polymeric phases with a low bonding density the elution order becomes $PhPh < BaP < TBN$. The separation factor for TBN and BaP has been used as a general guide to the shape selectivity of alkylsiloxane-bonded phases for the separation of rigid, non-polar compounds, particularly isomers (section 4.2.2.1). Values for the separation factor typically range from 0.3 to 2.2. Polymeric bonded phases that generally exhibit high shape selectivity have separation factor values < 1 and monomeric bonded phases of low shape selectivity > 1.7 . Similar general indications are observed for the separation of triphenylene and

o-terphenyl, see Table 4.22, with values of $\alpha_{T/O} > 3$ associated with significant shape selectivity for octadecylsiloxane-bonded phases and smaller values indicating low shape selectivity [662,675].

Residual silanol groups on chemically bonded silica-based phases have been associated with a number of undesirable interactions with polar solutes such as peak tailing and excessive or irreproducible retention. These problems are exemplified by the general difficulty encountered in the separation of amines and other strong bases, which includes many pharmaceutical compounds. An extensive literature has developed and a number of practical remedies have been suggested [253-256,676-679]. Improved stationary phases based on high purity silica with bonding chemistry designed to shield or minimize interactions between solutes and surface silanol groups has made an important contribution to this problem (section 4.2.2.1). A large number of methods have been proposed to evaluate the influence of silanophilic interactions on the separation of basic compounds, some of which are summarized in Table 4.21, with some typical results for different columns summarized in Table 4.22. The Engelhardt method classifies silica-based chemically bonded stationary phases as suitable for the separation of basic compounds if for the prescribed separation conditions four criteria are met: (1) aniline elutes before phenol; (2) the ratio of the asymmetry factor for aniline and phenol is < 1.3 ; (3) the selectivity factor for m- and p-toluidine < 1.3 ; and (4) N,N-dimethylaniline elutes before toluene. The Engelhardt test, like others based on the relative retention of weak bases, is rather undemanding with respect to the properties of modern phases prepared from high purity silica. These tests are generally not a good indication of the separation characteristics of strong bases that are often in a mixed ionization state in unbuffered mobile phases.

The difficulty in designing a comprehensive test for silanophilic interactions results from the heterogeneous properties of the silanol groups. Normal silanol groups are neutral, dipolar and strongly hydrogen-bonding. A small fraction of the silanol groups, most likely activated by trace metal ions embedded in the silica matrix, are strongly acidic and ionized even at low pH (section 4.2.1.1). These sites function as cation exchangers, and are probably responsible for the poor chromatographic properties of strong bases and contribute significantly to the low recovery and denaturing of proteins in reversed-phase chromatography. General tests of the ion-exchange capacity of reversed-phase packings are based on the separation factor for the bases benzylamine or aniline and phenol in a mobile phase buffered to a pH < 3 [662-664]. At this pH normal silanol groups are undissociated and only highly acidic silanols contribute to the retention of the amine by ion exchange. The separation factor for aniline and phenol at pH 2.7 for the stationary phases in Table 4.22 varies from 0.01 to 2.55 indicating a wide range of ion-exchange properties. There can be little confidence, however, that any of the various test methods provides a meaningful single scale of silanophilicity. Individual methods probably provide a composite assessment of the uncorrelated contributions from neutral polar interactions and ion exchange interactions weighted to different extents. There is only a poor correlation for the ranking of stationary phases by each method and for the same test using acidic and neutral mobile phases [664-666,676].

These methods fail in the unambiguous identification of the most suitable column packings for the separation of a wide range of basic compounds.

To identify specific stationary phases suitable for the separation of strong bases it was suggested that evaluations should be performed using several compounds covering a wide pK_a range, with hindered and unhindered ionizable groups and include compounds with multiple basic centers [254-256,678,680-682]. A general difficulty with these multiple probe tests is relating the results to the separation of different analytes with other experimental conditions. There is generally a strong dependence of the results on the pH, buffer composition and organic modifier type and concentration. These variables can have a strong influence on efficiency and asymmetry factors, which are often the chromatographic parameter used for column comparisons. Normalization of the results by subtraction of the expected efficiency obtained from interpolation of neutral compounds to the same retention factor from the observed efficiency allows additional peak broadening associated with silanophilic interactions independent of other contributions (e.g. extracolumn, packing heterogeneity, etc.) to be assessed [255]. A similar approach can be applied to the asymmetry factors. Cumulative frequency profiles are used as an aid for column comparison incorporating the results for all compounds into a single overlay plot. Since the concentration of strongly interacting sites on modern phases is expected to be low it is important not to invalidate the tests by overloading the column. For general quality evaluation of modern phases expected to have a low silanophilicity one or several strong bases such as pyridine, codeine, propranolol, amitriptyline, etc., are included in the test mixture [663,665]. An example of a comprehensive column quality test performed on four batches of the same column packing material is shown in Figure 4.34.

Residual silanol activity can have a beneficial effect on the separation of neutral polar compounds by supplementing hydrophobic interactions. It should not be construed that columns with high silanophilicity are undesirable for all chromatographic separations, just mainly for the separation of strong bases. They are the preferred choice for some separations.

Metal impurities embedded in the silica matrix or adsorbed on its surface provide strong adsorption sites for complexing (chelating) analytes capable of causing poor peak shapes and altered retention. Metal ions may also increase the acidity of adjacent silanol groups resulting in undesirable separation properties for strong bases. Two common tests for metal impurities include the DERT test and the metal factor test [664,670,683,684]. The DERT test compares the peak efficiency measured at the base of the two regioisomers 2,3- and 2,7-dihydroxynaphthalene with acetonitrile-25 mM ammonium acetate buffer pH 7.2 (25:75) as mobile phase. Only 2,3-dihydroxynaphthalene is capable of chelating with metals. For columns with a low metal content a DERT value close to 1 is expected. The metal factor test is based on the ratio of the asymmetry factors for 2,2'-bipyridyl and 4,4'-bipyridyl multiplied by 100 with methanol-water (49:51) as the mobile phase at 40°C. 2,2'-Bipyridyl complexes with metals ions having a charge higher than 2 while 4,4'-bipyridyl is non-complexing but has otherwise similar stationary phase interactions to 2,2'-bipyridyl. For columns with a

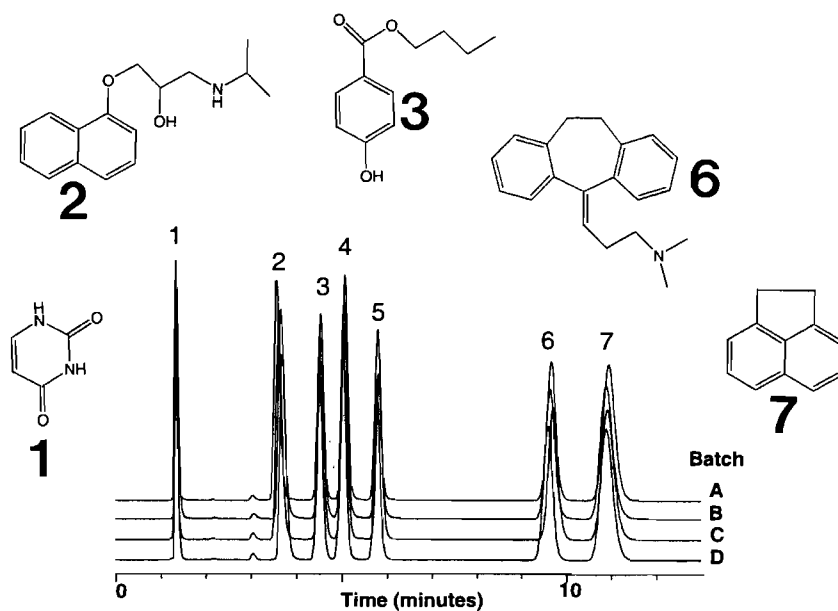


Figure 4.34. Overlay of the quality test chromatograms for four batches of SymmetryShield RP-8. Peaks: 1 = uracil; 2 = propranolol; 3 = butylparaben; 4 = dipropyl phthalate; 5 = naphthalene; 6 = amitriptyline; and 7 = acenaphthene. (From ref. [665]. ©Elsevier)

low metal content a metal factor value close to 100 is expected. Columns prepared from high purity silica generally have values < 200. Both tests are limited in that the primary source of metal contamination results from adsorption of metal ions from the mobile phase and system components (particularly frits and screens) in use and storage and does not, in general, accurately reflect the properties of the original column packing.

It is necessary to use the test methods presented in this section with some caution. They have proven invaluable for monitoring changes in a single column packing over time (manufacturing quality control) and in improving the synthesis of packing materials (laboratory optimization) but their use as an aid to column selection for method development is less certain. In particular, the various measures of silanophilicity probably reflect different relative contributions of multiple interactions rather than a single scale that expresses the full extent of these interactions. In addition, it should be noted that columns might deteriorate with use depending on their treatment. An old column of a type shown to perform well in a particular test in the literature may not live up to expectations, due to its altered state. Most of the test methods are based on isocratic separations. A few gradient methods have been described, largely for characterizing the properties of wide pore materials used for the separation of biopolymers [685,686]. The low retention of these materials is incompatible with some of the isocratic methods described in this section.

4.5.4 Effective Pore Size Distribution

The pore size distribution of a column packing material can influence its chromatographic properties through exclusion of high molecular mass solutes and excessive retention of solutes in micropores. It is common practice for manufacturers to quote the median pore size for the silica substrate used to prepare chemically bonded phases, which has two disadvantages. This value provides no indication of the pore size distribution, and it fails to take into account the reduction of the pore diameter due to partial filling of the pore volume by the bonded phase. The effective pore size distribution can be determined by size-exclusion chromatography using a series of poly(styrene) and hydrocarbon standards under separation conditions that minimize strong stationary phase interactions [102,687-689]. (Note that these methods really determine a pore volume rather than pore size distribution but are generally described as a pore size distribution). Each poly(styrene) standard has a characteristic random coil diameter in solution given by Eq. (4.39)

$$\Phi = 0.062(\text{MW})^{0.59} \quad (4.39)$$

where Φ corresponds to the diameter of the smallest pore in nanometers allowing unhindered access to a poly(styrene) standard of a given molecular weight (MW). Hydrocarbon standards are required to ensure that sufficient data points are available for the small pores and their molecular weight must be converted to a poly(styrene) equivalent value by multiplying by 2.3 for insertion into Eq. (4.39). The elution volume of individual standards is converted to the parameter R , the percentage of the total pore volume for which a given poly(styrene) standard has access, using Eq. (4.40)

$$R = 100[(V_E - V_0) / (V_M - V_0)] \quad (4.40)$$

where V_E is the elution volume of a standard able to explore some of the pore volume, V_0 the elution volume of a standard completely excluded from the pore volume, and V_M the elution volume of a standard able to explore the complete pore volume. The cumulative pore size distribution for the packing is then obtained by plotting R against $\log \Phi$ and fitting the data to a polynomial function for interpolation. Values of Φ corresponding to $R = 10, 25, 50, 75$, and 90% are then obtained from this plot. The value of Φ at $R = 50\%$, Φ_{50} , gives the mean pore diameter, and the difference between two Φ values is used to express the range (e.g. 80% of the pores have diameters between Φ_{90} and Φ_{10}). The effective pore size distribution for some typical column packing materials is given in Table 4.23. As would be expected large differences between the nominal pore size for the silica substrate and the effective pore size for the bonded phases are commonly found. Also the distribution of pore sizes can be very different for different stationary phases, which will be reflected in their chromatographic properties.

Table 4.23

Effective pore size distribution for reversed-phase sorbents determined by size-exclusion chromatography

Stationary phase	Nominal pore size	Pore size distribution (nm)				
		Φ_{10}	Φ_{25}	Φ_{50}	Φ_{75}	Φ_{90}
LiChrosorb RP-18	10.0	85.1	20.0	8.9		
Zorbax ODS	7.5			5.7	3.7	2.9
Vydac 218TP	33.0	116.2	56.2	25.1	11.0	1.5
Spherisorb ODS	8.0	24.5	12.6	7.1		
μ Bondapak C-18	12.5	41.7	21.4	10.0	5.0	3.0
Novapak C-18	9.0	263.0	75.9	6.8		
LiChrosorb RP-8	10.0	77.6	19.5	9.5		

4.5.5 Column Hold-Up Volume

The system hold-up volume is defined as the volume of mobile phase contained within the chromatographic system between the injector and the detector [690]. It can be determined by the elution volume of an unretained and unexcluded solute. The column hold-up volume is equivalent to the system hold-up volume less the extracolumn volume. The extracolumn volume is made up of contributions from injector, detector and connecting tubing volumes, which in a well-designed chromatographic system should be negligible compared with the column hold-up volume for conventional analytical columns. This may not be true for small bore and capillary columns, requiring an independent determination of the extracolumn volume for an accurate determination of the column hold-up volume (section 1.5.4). It would seem that the column hold-up volume should be an easy parameter to determine in liquid chromatography, but this is not the case. For packed columns, a problem arises from the difficulty of unambiguously defining the phase ratio.

The mobile phase is contained in the interparticle volume (the space between the particles) and in the volume inside the particles (intraparticle or pore volume), that is, in all those volumes not occupied by the fixed stationary phase [690-693]. The interparticle volume can be divided into two parts, that portion of the volume containing the moving phase, and that volume close to the points of contact between the particles and away from the flowing stream that is essentially static. The mobile phase within the pores is stagnant. The composition of mixed mobile phases trapped in the pores is not constant, since one or several components of the mobile phase can become selectively adsorbed or associated with the stationary phase resulting in a region of altered composition in the vicinity of the stationary phase. For kinetic column characteristics, such as the linear velocity and band broadening phenomena, it is the volume of the moving mobile phase that is important. In this case the hold-up volume is essentially equivalent to the volume occupied by the moving portion of the interparticle volume. The thermodynamic hold-up volume is used to calculate the retention factor, separation factor, etc. It includes all those portions of the mobile phase (both static and moving) that have the same composition. The difficulty of defining the thermodynamic hold-up volume arises from two sources. It is difficult to define the volume of solvated stationary phase along the pore wall. This volume will also depend on the composition of the mobile phase.

Because of exclusion effects solutes of a different size or containing charges will have available to them different phase volumes. In the thermodynamic sense a column may have a range of solute-specific hold-up volumes reflecting the phase volumes accessible to individual solutes.

In a detailed study of a 25 cm x 4.6 mm I.D. column packed with Zorbax C8 (5 μm particles and a surface area 330 m^2/g) with methanol-water (1:4) as mobile phase it was shown that 33% of the column volume was occupied by the fixed stationary phase [690]. The pore volume (21 % of the column volume) consisted of 21% adsorbed solvent and 79% bulk solvent. The interparticle volume (46% of the column volume) consisted of 73% moving phase and 27% static phase. It is not easy, however, to determine the various column volume elements, and generally kinetic and thermodynamic parameters have been determined for inadequately defined phase ratios and may not be correct in the absolute sense.

A large number of experimental methods have been proposed to estimate the column hold-up volume without any single method emerging as a preferred choice [691-698]. Comments and opinions on comparative studies have generally resulted in contradictory conclusions. The principal experimental methods include static weighing procedures; the determination of the elution volume of a solvent disturbance or system peak obtained by injecting a single component of a mixed mobile phase; determination of the elution volume of a neutral and unexcluded solute that gives the lowest elution volume; determination of the elution volume of an easily detected ion; determination of the elution volume of an isotopically labeled component of the mobile phase; and mathematical procedures based on linear regression of the elution volumes for members of a homologous series. The total porosity of the column can be determined by successively filling the column with two solvents of different density (e.g. methanol and carbon tetrachloride) and then weighing it after each filling [690,692]. This method is tedious and provides a value for the maximum volume accessible to the mobile phase. It ignores the possibility that the stationary phase is solvated by the mobile phase reducing the column volume occupied by the mobile phase. One of the simplest methods of determining the hold-up volume when mixed mobile phases are used is to inject one of the pure mobile phase components and determine the elution volume of the disturbance peak [692-695]. However, the disturbance peak is not always easy to identify and its position is coupled to the mobile phase composition. The disturbance peak corresponds to the hold-up volume only when the packing more or less equally sorbs the mobile phase components. By far the most popular approach for reversed-phase columns is to determine the elution volume of an ionic or organic compound that is believed to be unretained, such as sodium nitrate, potassium bromide, potassium dichromate, uracil, thiourea, acetone, dimethylformamide or fructose [692-697]. These methods are basically limited by partial or complete exclusion from the pore volume of the packing by size or ion exclusion effects and by retention of some test solutes by the stationary phase. For neutral solutes retention by the stationary phase is the most common problem and manifests itself in the form of elution volumes that vary with the mobile phase composition. Ionic compounds are affected mainly by ion exclusion.

Their elution behavior is influenced by the amount of sample injected and the presence of background electrolyte in the mobile phase. At low sample concentrations in non-buffered mobile phases, a salt is excluded from the pore volume of the packing, presumably due to electrical charges on the phase surface [690,694,697]. Alternatively, if a buffered mobile phase or injection of a concentrated salt solution into a non-buffered mobile phase is used, then exclusion of the salt from the pore volume is minimized and constant elution volumes are obtained. Injection of a single isotopically labeled mobile phase component results in a poor estimate of the hold-up volume due to the disturbance of the stationary phase solvation layer resulting in retention of the labeled component [691,693-695]. This can be accounted for by labeling all components used in the mobile phase and using their characteristic elution volumes to calculate the hold-up volume [691,695]. This process is time consuming and usually requires off-line detection by a scintillation counter. Mathematical methods of determining the hold-up volume are based on a linear or non-linear relationship between the logarithm of the retention factor and carbon number for members of a homologous series (e.g. 2-alkanones, 2-phenylketones, 1-nitroalkanes) [692,693,698]. This method requires high quality retention data for a minimum of 4 or 5 standards to obtain reasonable statistics for the model fit. Different models, different homologous series or different members from the same homologous series, can result in (slightly) different values for the hold-up volume.

When a value for the hold-up volume is required expediency tends to take precedence over exactness. Most of the methods discussed in this section will provide a reasonable estimate of the hold-up volume, when used within their limitations, but none can be stated to determine the correct value. It is not a simple matter to formulate a solution to this problem. In the absence of improved methods for determining the hold-up volume, it is important that the method used for its determination is reported along with thermodynamic and kinetic properties derived from its use.

4.6 REFERENCES

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Chapter 5

Instrumental Aspects of Liquid Chromatography

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5.1 INTRODUCTION

Modern liquid chromatography requires instrumentation capable of accommodating columns of different sizes with a range of optimum operating conditions. Typical features include high-pressure operation, accurate preparation of mobile phase compositions, stable mobile phase flow rates and on-line detection, all under conditions that preserve the high peak efficiency of the column. A block diagram of a suitable instrument for modern liquid chromatography is shown in Figure 5.1. A solvent delivery system consisting of reservoirs, mixers, valves, pumps, dampers and flow and pressure controllers is responsible for delivering the desired mobile phase composition to the head of the column as a continuous and (nearly) pulse free stream at a known pressure and volumetric flow rate. An injection valve is connected to the head of the column for loading the sample solution under ambient conditions and then inserting a known volume of the sample into the fully pressurized mobile phase flow. The separation is recorded continuously on the low-pressure side of the column using an on-line detector with a small operating volume, high sensitivity and a fast response. To achieve optimal control of the individual components the operation is supervised and managed by a computer or several microprocessors.

The basic components of a liquid chromatograph cover a wide range of sophistication, features and cost [1-5]. Most systems are modular to provide maximum operating flexibility and frequently fully automated. Advanced materials allow the fabrication of instruments from non-metallic components for enhanced biocompatibility and corrosion resistance in life science and ion chromatography applications. Continuing improvement in design and electronic control provides for ease of use with a wider range of column diameters from semipreparative to packed capillary columns at close to optimum separation conditions. Interest in small-diameter columns stems from their lower

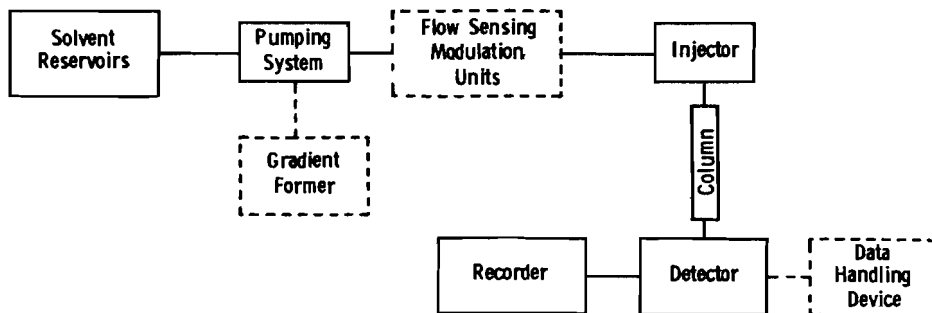


Figure 5.1. Block diagram of a modern liquid chromatograph. Dotted lines refer to optional components.

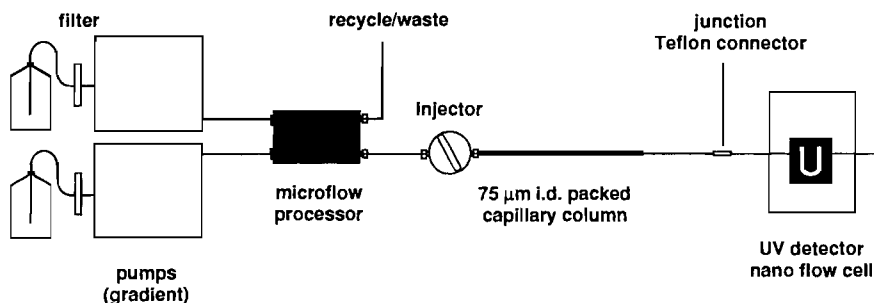


Figure 5.2. Schematic diagram of a standard liquid chromatograph modified for use with a packed capillary column. Typical pump settings are 300–400 μl / min with flow splitting of 1:2000 to give a column flow of 150–200 nl / min. The microinjection valve has a 40 nl internal loop and an additional 1:10 split creates an injection volume of 2–3 nl (larger volumes can be injected by on-column focusing with gradient elution separations). The detector uses a U- or Z-shaped flow cell with a 3 nl volume and 8 mm path length. Fused-silica capillary tubing with an internal diameter $\leq 20 \mu\text{m}$ and zero-dead-volume connectors are used for column connections. (From ref [8]. ©American Chemical Society).

solvent consumption, greater mass sensitivity when sample amounts are limited, and ease of interfacing to other separation and detection techniques. In addition a number of soft ionization techniques used for the identification of biopolymers by mass spectrometry are facilitated by the use of low mobile phase flow rates (these studies account for the majority of practical applications using packed capillary columns) [6–8]. These columns are also the most demanding with respect to instrument design and typically use standard instruments that are adapted to provide low flow and minimal extracolumn band broadening [6–9]. A typical arrangement is shown in Figure 5.2. Modifications include addition of a microflow processor for flow splitting, a microinjection valve for handling sub- μl sample volumes, a longitudinal U- or Z-shaped nanoflow cell for sensitive UV detection, and connecting capillaries of small internal diameter to minimize peak dispersion. Instrumentation for ultrahigh-pressure liquid chromatography has been described to overcome the limits of separation speed and peak capacity that result from the pressure operating limit of conventional instrumentation when small-diameter particles (1–2 μm) are used in long capillary columns [10–14]. Instruments for operation at the low μl / min flow rates at pressures up to 60,000 p.s.i. (4,000 atm) characteristic of these separations are not commercially available.

A detailed account of the contribution of injection, detection and connecting volumes to extracolumn band broadening was presented in section 1.5.4. It is generally accepted that instrumental contributions to band broadening should not exceed 10% of the column variance. This criterion corresponds to a loss of 10% of the column efficiency and about 5% in the column resolution. A Gaussian peak eluting from a column will occupy a volume equivalent to 4σ units. In which case the acceptable limits for extracolumn variance and volume for typical columns can be established using the properties of an unretained peak as the worst case or limiting condition, Table 5.1. Extracolumn volumes, which include the detector and injector volumes, should be

Table 5.1

Typical operating conditions for packed columns with different diameters

Column type	Column diameter (mm)	Flow rate	Injection volume	Peak volume ($k \approx 0$) (μl)	Peak variance (μl) ²
Conventional	4-5	0.5-2.0 ml / min	20 μl	20-200	25-2500
Small bore	1-2	50-200 μl / min	1-4 μl	1-12	1-25
Capillary	0.25-0.5	2-5 μl / min	0.1-1.0 μl	0.2-0.8	10^{-3} - 10^{-1}
	0.05-0.25	0.1-2 μl / min	2-100 nl	0.008-0.2	10^{-6} - 10^{-3}

less than about 20 μl for conventional packed columns, 1 μl for small bore packed columns, and about 100 nl for packed capillary columns. It should be feasible to manufacture instrument components with volumes around 100 nl and above, but difficult to miniaturize these components further. Consequently, instrumentation that allows the full potential of conventional and small bore columns is well within reach. For packed capillary columns, especially those of small internal diameter, the limits to miniaturization by manufacturing technology is reached and operating conditions for these columns are often a compromise between convenience and performance.

5.2 SOLVENT DELIVERY SYSTEMS

The solvent delivery system is responsible for delivering the pressurized mobile phase with the desired composition and chosen flow rate to the head of the column. To achieve this goal a number of components work together under the supervision of the system computer or microprocessors to achieve the tight specifications typical of a modern liquid chromatograph, Table 5.2 [15-19]. Suitable tests for performance evaluation of solvent delivery systems are briefly described at the end of section 5.2.2. In retention terms a relative standard deviation of better than 0.15% for retention factors under normal operating conditions is expected.

5.2.1 Solvent Reservoir and Degassing

The solvent reservoir is a storage container made of a material resistant to chemical attack by the mobile phase. In its simplest form a glass jug, solvent bottle or Erlenmeyer flask with a cap and a flexible hose connection to the pump is adequate. The connecting hose is terminated on the solvent side with a 2- μm porous filter to prevent suspended particle matter from reaching the pump. In more sophisticated instruments the solvent reservoir may also be equipped for solvent degassing [20]. Degassing may be required for efficient pump and detector operation, particularly for aqueous organic mobile phases and for gradient forming devices using low pressure mixing. Degassing, in this case, is used to prevent gas bubble formation when different solvents are mixed or the mobile phase is depressurized. Dissolved oxygen in the mobile phase is a source of reduced sensitivity and poor baseline stability for ultraviolet, fluorescence and

Table 5.2

Typical specifications for a solvent delivery system for use with conventional diameter packed columns

Property	Specification
Flow rate range	0.01-10 ml / min
Flow rate accuracy	< 1% at 1.0 and 4.0 ml / min
Flow rate precision	< 0.2% at 0.5 ml / min
Composition accuracy	< 1% at 1.0 ml / min with 5-95 % (v/v) solvent mixtures
Compositional precision	< 1% at 1.0 ml / min
Dwell volume	0.5-0.8 ml (t_D in Figure 5.3B)
Pressure range	to 6,000 p.s.i (420 atms)
Pressure pulsation	< 1% at 1.0 ml / min

Description of some properties of the solvent delivery system

Resetability	The ability to reset the pump to the same flow rate repeatedly.
Flow rate accuracy	The ability of the pump to deliver exactly the flow rate indicated by a particular setting.
Pulsation	Flow changes sensed by the detector as a result of pump operations, such as piston movement and check valve operation.
Short term precision	The accuracy of the volume output of the pump over a few minutes.
Drift	Measure of the generally continuous increase or decrease in the pump output over relatively long periods (e.g. hours).

Pressure conversion factors

$$1 \text{ bar} = 14.5 \text{ p.s.i.} = 0.9868 \text{ atm} = 1.02 \text{ kg / cm}^2 = 0.1 \text{ MPa.}$$

electrochemical detectors [2,4,20,21]. Solvated oxygen complexes absorb significantly in the low wavelength UV region, 190-260 nm, and changes in the mobile phase oxygen concentration leads to detector baseline drift, random noise, a loss of sensitivity, and a large baseline offset at high sensitivity. Oxygen can cause intersystem energy transfer from the excited state of fluorescent compounds resulting in "quenching" of the fluorescence signal through this non-radiative decay pathway. Oxygen removal to very low levels is an absolute requirement for electrochemical detection in the reduction mode, since the oxygen reduction current contributes deleteriously to the residual current and detector noise. In the above cases temperature fluctuations and oxygen concentration may be coupled together in reducing detector baseline stability at high sensitivity.

Degassing can be carried out by applying a vacuum above the solvent, heating with vigorous stirring, ultrasonic treatment, sparging with helium or by using a gas permeable membrane. Only sparging with helium and gas permeable membranes are suitable for routine use. Helium sparging removes about 80-90% of the dissolved air within 10 minutes by drawing the unwanted dissolved gases from solution as they equilibrate with the helium bubbles. Since helium has a very low solubility in common solvents, after sparging the solvents are nearly gas-free. Redissolution of unwanted gases is prevented by continued sparging with helium or by maintaining the mobile phase in a helium atmosphere. Particular care is needed when sparging with helium to prevent back diffusion of air into the solvent reservoir against the much lighter helium

gas. This includes the exclusion of Teflon (or related plastic) tubing, permeable to air, from the solvent delivery system. On-line solvent degassers based on gas permeable membrane technology are now commercially available. The solvent flows through a narrow-bore polymer tube housed in a chamber connected to vacuum. Removal of air is very efficient from most solvents without consuming expensive helium.

An alternative method for oxygen removal is catalytic reduction over a platinum-on-alumina catalyst in the presence of methanol [22,23]. A short column packed with the catalyst is inserted between the pump and injector. Provided that the mobile phase contains about 1% methanol (or more) oxygen is very efficiently reduced producing formaldehyde and formic acid. It is necessary to ensure that these products do not react with or degrade the sample. An in-line chelation column placed between the pump and injection valve was suitable for the removal of metal impurities from the mobile phase [24].

5.2.2 Pumps

The types of pumps used in modern liquid chromatography can be divided into two categories according to the mechanism of dispensing the mobile phase: constant pressure pumps (e.g. gas displacement and pneumatic amplifier pumps) and constant volume pumps (e.g. reciprocating-piston and syringe pumps) [1-4,25]. For analytical separations only the constant volume pumps are important. Pneumatic amplifier pumps are used for slurry packing columns and in some preparative-scale liquid chromatographs. The pneumatic amplifier pump uses a large bore gas piston to drive a liquid piston of smaller size in a dual chamber configuration. The pressure on the liquid piston is proportional to the gas pressure and the area ratio of the two pistons. The pneumatic amplifier pump is equipped with a power-return stroke that permits rapid refilling of the empty piston chamber with mobile phase. This pump can provide high pressures and relatively high volumetric flow rates. For use with packed capillary columns, a novel solvent delivery system that affords a constant pulse-free flow in the nl / min to μ l / min range have been described [26,27].

Syringe pumps employ solvent displacement by a mechanically controlled piston advancing at a constant speed in a fixed volume chamber. The pump output is relatively pulse free, very high pressures can be obtained, and gradient and flow programming are quite straightforward, at least in principle. Since solvents are compressible, a finite time is required before the volumetric flow rate is constant at high pressures. A combination of high cost, limited solvent reservoir capacity and problems with solvent compressibility have resulted in the more or less complete replacement of syringe pumps by reciprocating-piston pumps for use with columns of conventional internal diameters. Solvent compressibility problems are less significant for pumps with small reservoir volumes operated at low flow rates. Thus, syringe pumps with cylinder volumes of 10-50 ml are sometimes used in instruments designed only for small bore and packed capillary column operation, where typical flow rates are less than 200 μ l / min. Syringe pumps are also commonly used in instruments designed for open tubular column supercritical fluid chromatography.

Most general-purpose pumps used today are of the single or dual reciprocating-piston type. Each stroke of the piston displaces a small volume (typically 10-400 μL) from a pump chamber equipped with inlet and outlet check valves. During the intake (fill) stroke, the piston is withdrawn from the cylinder, lowering the pressure upstream from the inlet check valve. The inlet check valve opens, and liquid flows into the cylinder. The outlet check valve closes, because the system pressure exceeds the cylinder pressure. On the delivery stroke, the piston moves into the cylinder, the inlet check valve closes, the outlet check valve opens, and liquid flows to the column. A piston seal prevents solvent from leaking out of the chamber during piston motion. Pistons and check valve balls are usually made from sapphire or ruby, and the pump head from stainless steel. For high salt or extreme pH mobile phases pump heads made of non-metallic parts are required. PEEK is the most popular alternative to stainless steel. It is a strong and relatively inert plastic that can be machined into complex shapes, almost as easily as metal, and is suitable for use at pressures up to about 5,000 p.s.i. Other polymers such as Teflon are restricted to pressures of only about 2,000 p.s.i. Some pump heads are made from titanium, an expensive metal that is not completely free of corrosion problems, as well as a source of mobile phase metal contamination.

The output from a reciprocating-piston pump is inherently unstable. To smooth the pulsed flow from a single-piston pump an asymmetrical cam is used to drive the piston, which speeds up the piston movement during the refill stroke, and produces a uniform delivery over most of the cycle regardless of flow rate. With a dual-head reciprocating-piston pump both chambers are usually driven by the same motor through a common cam, such that the two pistons are 180° out of phase. As one chamber is emptying the other is refilling; thus the two flow profiles overlap, leading to partial cancellation of the peaks and troughs in the total flow output. An alternative design for a dual-head, reciprocating-piston pump uses the second piston as a pulse compensator. It operates in a valveless chamber connected in series to the main chamber. The compensating piston withdraws at a slower rate during the delivery stroke of the main piston, accumulating liquid to be dispensed when the main chamber is refilling.

Reciprocating-piston pumps deliver a constant flow at a fixed backpressure. At high pressures some minor flow variations may arise due to the compressibility of the mobile phase. Some instruments incorporate a flow controller which provides a fixed backpressure for the pump to work against, independent of the column backpressure. The influence of pressure fluctuations, solvent compressibility, and solvent viscosity on the volumetric output of the pump is thereby eliminated. Reciprocating-piston pumps can provide continuous solvent delivery, fast solvent change over, gradient elution compatibility, and have low maintenance requirements.

In general terms, accurate flow control with a minimum of pulsation and drift is the hallmark of a good pump. Flow reproducibility can be evaluated from changes in the elution time of an unretained solute over time or by collecting the mobile phase for a set time and weighing the collected fraction. Mobile phase composition accuracy can be determined by replacing the column with a length of capillary tubing and measuring the step height increases on the detector trace for mixing two solvents (or the same

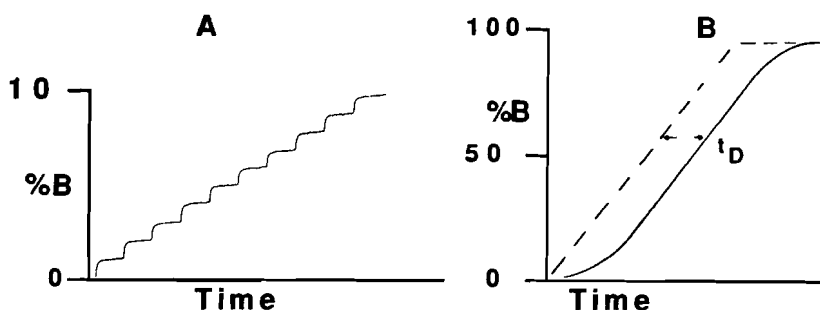


Figure 5.3. An example of the proportioning accuracy of a reciprocating-piston pump in the composition range 0-10% B in 1% increments (A). The influence of the design of the solvent delivery system on gradient shape (B). The Broken line describes the set gradient and the solid line the gradient delivered to the column inlet. The dwell time is marked as t_D .

solvent) in which a small amount ($< 1\%$) of a UV absorbing substance (e.g. acetone, thiourea, etc.) is added to one of the solvent reservoirs. Suitable solvent increments are 10% for 0-100% solvent B and 1% for 1-10% solvent B for a binary solvent mixture AB, Figure 5.3. Most pumps have difficulty in mixing a few percent of one solvent in another and the largest variations in composition accuracy can be expected for solvent mixtures containing only a few percent of one of the solvents. The contribution of the solvent delivery system to detector baseline noise can be determined by the difference in baseline disturbance for measurements made with and without flow through the detector cell. Additional tests of the solvent delivery system are summarized in [15-18] and some typical specifications for reciprocating-piston pumps are given in Table 5.2.

5.2.3 Pulse Dampers

Some reciprocating-piston pumps use a pulse damper in series with the pump head(s), and most also use some sort of electronic feedback control to vary the pump motor speed within each cycle to further reduce pulsations, as well as to maintain accurate flow as the column backpressure varies. Mechanical pulse dampers store energy during the pressurizing stroke and release it during the refill stroke. Pulse dampening has been achieved using a variety of devices, such as bellows or coiled tubes, often in conjunction with a fluid or gas ballast reservoir. One common pulse damper uses a flattened length of PTFE tubing immersed in a degassed compressible liquid. The flexibility of the tubing and the compressibility of the fluid thus absorb any pressure fluctuations in the solvent stream. Alternatively, the flow output can be passed over a thin, flexible, steel membrane, one side of which is in contact with a compressible fluid.

An alternative approach to providing an accurate and essentially pulse-free flow over a wide range of different solvent compositions and pressures is provided by a combination of flow and pressure feedback loops. A monitoring circuit regulates the motor speed to maintain a constant pressure determined by a pressure transducer

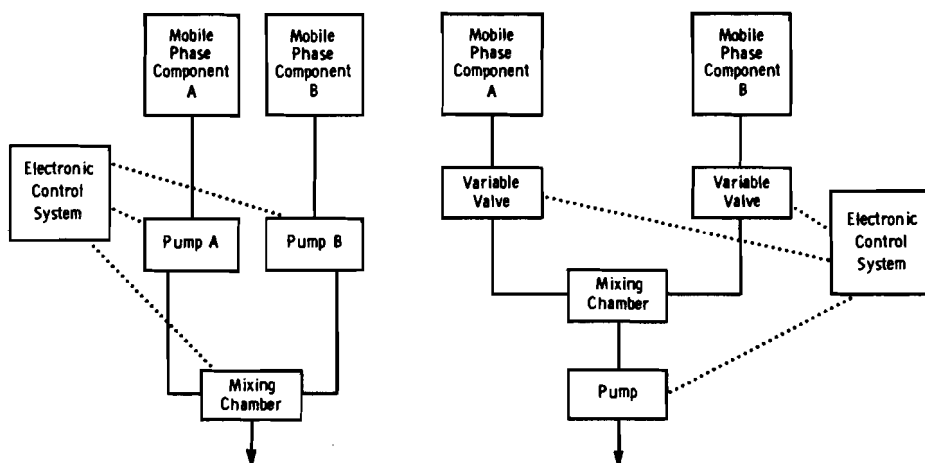


Figure 5.4. Two methods for generating binary solvent gradients.

downstream of the pump. Simultaneously an encoder wheel attached to the motor drive regulates the motor revolutions to a near constant value

5.2.4 Composition Gradients

A mobile phase composition gradient is formed by mixing two or more solvents, either incrementally or continuously, according to some predetermined gradient shape. The most frequently used gradients are binary solvent systems with a linear, concave or convex increase in the percent volume fraction of the stronger solvent. Gradient devices are usually classified into two types, depending on whether the solvents are mixed on the low- or high-pressure side of the pump, Figure 5.4. Low-pressure mixing devices use a manifold containing up to four time-proportioning valves, each connected to a different solvent. A microprocessor controls the time each proportioning valve is open and for maximum accuracy synchronizes the operation of the proportioning valves with the movement of the piston [1-3,28]. Low-pressure mixing is little influenced by solvent compressibility effects and can completely eliminate errors connected with thermodynamic volume changes due to mixing of the solvents. The main problem with low-pressure mixing is that it is susceptible to bubble formation because the solvents are mixed at atmospheric pressure, particularly in the absence of on-line degassing. The majority of modern liquid chromatographs use low-pressure mixing for gradient formation.

For high-pressure mixing each solvent is pumped separately in the proportions required by the gradient into a mixing chamber before being delivered to the column. Solvent compressibility and thermodynamic volume changes on mixing may influence the accuracy of the composition delivered to the column. A significant disadvantage of high-pressure mixing is the need for a separate pump for each solvent used.

The mixer in a gradient system is required to dampen fluctuations in mobile phase composition caused by the fact that the solvent delivery from reciprocating-piston pumps occurs in discrete steps. Effective reduction of detector baseline noise caused by variations in mobile phase composition requires a mixer that has a large volume relative to the piston volume of the pump. Typically, a compromise must be made between the desire to have a fast response to solvent composition changes at the column inlet and detector noise caused by short-term composition variations. Generally, homogeneous mixing can be achieved either by static flow mixing devices (open or packed tubes) or by actively stirred dynamic mixing chambers of small volume.

Differences in gradient elution separations that arise from the use of different equipment are due mainly to differences in the dwell volume, mixing volume, and the use of malfunctioning or poorly designed equipment [29,30]. Blank runs with a UV-absorbing compound added to the mobile phase can be used to measure the actual gradient delivered to the column compared with the gradient selected. With the sample injector connected directly to the detector (column removed) and methanol containing about 0.01% acetone in solvent reservoir A and methanol containing 0.1% acetone in solvent reservoir B the UV detector wavelength and sensitivity is adjusted to obtain about 90% full-scale response for solvent B. The blank gradient is then run from 0 to 100% B (or other desired range) over an appropriate time (e.g. 20 min) with the normal column flow rate. A typical example for a linear gradient is shown in Figure 5.3, the broken line representing the programmed gradient and the solid line the gradient delivered to the column inlet. Gradient delay and rounding are observed to varying degrees in all gradient systems. The gradient delay time, marked as t_D in Figure 5.3, is determined by the volume of the mixer plus all volume elements between the mixer and column inlet (including the pump for low pressure gradient mixing). For modern instruments designed for conventional packed column use, typical dwell volumes are 0.2-8.0 ml (often higher for instruments with an autosampler), corresponding to a dwell time of 0.2-8.0 min at a flow rate of 1 ml / min. Differences in the dwell time are responsible for variation in the time taken for different instruments to deliver a change in mobile phase composition to the column inlet. The rounding of the ends of the gradient is related to the volume of the mixer. The programmed gradient and the experimental gradient will be most alike when the dwell volume is as small as possible but the physical layout of the solvent delivery system and the need to dampen fluctuations in the mobile phase composition dictates that a compromise size is used in practice. Alternatively, the effect of dwell volume on the separation can be eliminated if the sample injection is delayed by the time (dwell time) required for the gradient to reach the column inlet. It is important that the column hold-up volume and the gradient dwell volume are known to accurately relate solvent composition to the elution position of peaks in gradient elution chromatography (section 4.4.6).

Variability in retention volumes and peak widths in gradient elution chromatography is due primarily to the limits of precision and reproducibility of the mobile phase composition, the flow rate, and the column temperature. Random or systematic deviations from the preset mobile phase composition and flow rate are caused by

imperfect functioning of the mechanical parts of pumps (plungers, valves, seals, etc.) or the system controller. Deviations from the instrument gradient can also occur due to solvent demixing, resulting from preferential adsorption of the stronger solvent by the stationary phase.

5.3 SAMPLE INLETS

The sample introduction system must be capable of introducing a known and variable volume of sample solution reproducibly into the pressurized mobile phase as a sharp plug without adversely affecting the efficiency of the column. The superiority of valve injection has been adequately demonstrated for this purpose and is now universally used in virtually all modern instruments for both manual and automated sample introduction systems [1,2,7,31,32]. Earlier approaches using septum-equipped injectors have passed into disuse for a several reasons, such as limited pressure capability, poor resealability, contamination of the mobile phase, disruption of the column packing, etc., but mainly because they were awkward and inconvenient to use compared with valves. For dilute sample solutions volume overload restricts the maximum sample volume that can be introduced onto the column without a dramatic loss of performance. On-column or precolumn sample focusing mechanisms can be exploited as a trace enrichment technique to enhance sample detectability. Solid-phase extraction and in-column solid-phase microextraction provide a convenient mechanism for isolation, concentration and matrix simplification that are easily interfaced to a liquid chromatograph for fully or semi-automated analysis of complex samples (section 5.3.2).

5.3.1 Valve Injection

A typical injection valve consists of a rotating seal (or rotor) and a fixed body with a number of external ports for attachment of sample loops, fill ports, waste lines, and column and pump connections, Figure 5.5. External sample loops are constructed from stainless steel tubing of various diameters, and are connected to the valve body using standard compression fittings. Rotation of the valve core connects different ports in series allowing the sample loading operation to be separated from the flow path of the mobile phase and then for the sample-containing loop to be rapidly inserted into the mobile phase flow path. In the load position, the sample is introduced into the loop at low pressure by a calibrated syringe fitted with a blunt-tip needle. A popular variation of the above design employs a center port for sample loading connected internally to the sample loop. The valve body is typically made of stainless steel, or Hastelloy or PEEK for improved corrosion resistance. The rotor or inner portion of the valve is typically made of polyimide or PEEK. All valve materials that come into contact with the sample or mobile phase must be inert and nonsorptive of the sample to avoid memory effects. Typical injection valves are leak tight to about 7-10,000 p.s.i at room temperature (pressure limits are lower at higher temperatures). Valve automation is achieved by

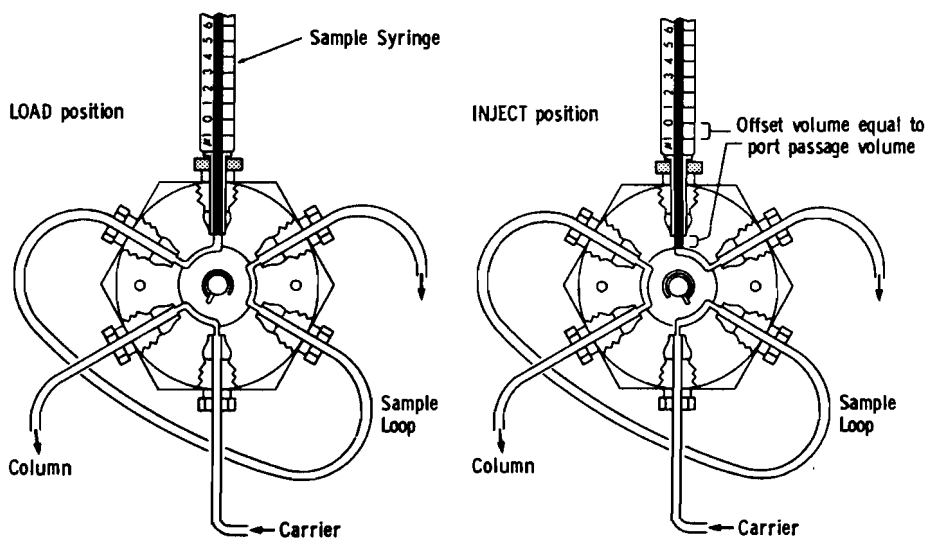


Figure 5.5. Sample injection valve showing the valve configuration in the load and inject position. (Reproduced with permission from Valco Instruments, Inc.).

using electric or pneumatic actuation and generally provides improved accuracy and precision compared to manual injection.

The sample is loaded at atmospheric pressure into an external or internal loop, or groove in the valve core and introduced into the mobile phase stream by a short rotation of the valve. The volume of sample injected is normally varied by changing the volume of the sample loop or by partially filling a sample loop with a fraction of its nominal volume. External sample loops have volumes from about $5\ \mu\text{l}$ up to about 5 ml, although typical injection volumes for conventional diameter columns are 10–50 μl . Injections from 1 μl to about 40 nl require micro-injection valves equipped with replaceable internal loops [7,32–34]. Injection volumes less than about 40 nl are performed by positioning a split vent between the injector and the column. Typical injection volumes that preserve column efficiency for packed columns of different internal diameters are summarized in Table 5.1. For packed capillary columns with internal diameters $< 0.2\ \text{mm}$ direct injection will usually require the use of a split vent to minimize volume overload unless on-column focusing is possible. Injection volumes about 5 times larger than those indicated in Table 5.1 are sometimes used to increase sample detectability but with some decrease in the column separation power.

The principal valve injection techniques are complete fill or partial fill with respect to the loop volume, timed injection and on-column focusing. The volume injected for the complete fill technique is determined by the size of the loop, which has been overfilled to completely displace the mobile phase. As a sample is introduced into the loop, it displaces the mobile phase ahead of it out of the loop. As this process proceeds, the

leading edge of the sample becomes diluted because the fluid traveling through the loop has a parabolic flow profile as a result of laminar flow. This causes the core of the fluid stream to be moving faster than the edges in the vicinity of the loop wall. As a consequence, to fill the loop completely and homogeneously with sample, the sample volume must be at least twice the volume of the loop to displace the entire diluted sample formed initially as the sample is introduced. Laminar flow also causes the sample band to be diluted as it is pushed from the loop when inserted into the mobile phase stream. About 5-10 loop volumes are required to completely displace the sample from the loop. Thus the entire sample does not enter the column as a plug, but with an exponential decay injection profile that contributes to the dispersion of the separated sample bands in an adverse manner. The sample plug profile depends on the loop dimensions and the diameter of passages connecting the loop to the rest of the system, the mismatch of diameters when two passages connect, and the inherent asymmetry in the injection plug profile due to the fact that the front boundary of the sample plug does not pass through all of the passages experienced by the rear boundary. At some point it is counter productive to reduce sample volumes further. The peak height continues to decrease affecting sample detectability without a commensurate reduction in peak width. The injection volume at which this occurs depends on the design of the injector but is easily observed in the range of small injection volumes (< 100 nl) employed with packed capillary columns with diameters < 0.2 mm [33].

When partial fill injection is used the volume injected is determined by the amount dispensed from the loading syringe. This volume should be less than half the sample loop volume, to prevent the diluted front of the sample band from leaving the loop as the sample is being loaded. The connections of most valve injectors are made so that sample flows out of the loop during injection in a direction opposite to that during loading. In the partial fill technique, this minimizes dispersion of the sample plug because sample does not pass through the entire length of the loop. Thus a large loop can be used to accommodate a wide range of sample volumes. This approach is often used with autosamplers that allow series injections of different volumes.

The timed-injection technique uses an actuator to move the valve from the load to inject position only long enough for the mobile phase to transfer the desired volume out of the loop. The loop volume must be at least twice the selected injection volume because the sample plug is displaced from the loop under laminar flow conditions. The timed-injection technique can be used to cut off the tailing portion of the sample injection plug to retain maximum column efficiency. Injection variances close to theory can be obtained. However, provided that the loop volume is not more than about 20% of the peak volume of the first peak of interest in the chromatogram, the shape of the injection profile can be ignored for practical purposes. This is the usual case for typical injection volumes used with conventional diameter columns, but is a more significant problem as the column diameter is decreased.

It is generally recommended that whenever possible samples are prepared for injection in the same solvent mixture as used for the mobile phase, or in a solvent that is weaker than the mobile phase but miscible with it. Injection solvents stronger than

the mobile phase can cause variations in retention time and peak widths as a function of the injection solvent composition, and in the case of fairly large injection volumes, split peaks may also be observed. These effects are caused by variations in the penetration length of the sample zone at the head of the column due to displacement of the sample from the stationary phase by the injection solvent plug during the time it is diluted by the weaker mobile phase. When the sample is injected in a solvent much weaker than the mobile phase on-column focusing allows much larger injection volumes to be used than would otherwise be possible without inducing volume overload of the column [7,33-38]. For isocratic conditions the maximum volume that can be injected without additional peak broadening using a weak injection solvent, V_{oct} , is related to the maximum injection volume of an unretained peak without on-column focusing, V_{io} , by the approximate relationship

$$V_{\text{oct}} = V_{\text{io}}(1 + k_0)^2 / (1 + k) \quad (5.1)$$

where k_0 is the retention factor of a solute with the sample solvent as mobile phase and k is the retention factor of the solute when eluted by the mobile phase. The above relationship assumes that mass overload, displacement effects and analyte matrix interactions are unimportant. To obtain a significant increase in the injection volume by on-column focusing requires $k_0 > k$ and also that k is small. Enrichment factors of several hundred are possible. This method has been used with small bore and packed capillary columns to overcome the loss of detection sensitivity due to the volume or mass limits of normal-size injection techniques. It is also equally applicable to conventional diameter columns but limited detection sensitivity is less of a problem in this case, and for trace enrichment, coupled-column systems are often a better practical alternative (section 5.3.2). The use of short precolumns for the focusing step allows a higher level of sample matrix contamination to be tolerated with real samples as well as selective sampling by transferring to the separation column only a fraction of the concentrated sample containing the components of interest. Independent of the column diameter the use of a separate precolumn allows much higher flow rates to be used for the sample loading step, which can be quite considerable and inconvenient at the normal flow rates for small diameter columns.

Full automation of separations by liquid chromatography requires automation of the sample introduction process. This includes automation of scheduled injections and automation of sample processing when required to isolate the analytes of interest from the sample in a form suitable for separation and detection. An example of the latter approach is on-line solid-phase extraction-liquid chromatography (SPE-LC) discussed in section 5.3.2. Automation of time scheduled injections increases accuracy and precision by removing human error [1,18,39]. Typical precision for manual valve injections is about 0.5% for complete-fill and 1-2% for partial-fill loop injections. Typical precision for automated injection is about 0.25% for complete-fill and 0.5% for partial-fill loop techniques.

Autosamplers usually hold several samples contained in septum capped vials in a carousel or tray that serves as a fixed address for each sample. These samples are

usually held for an extended time at room or subambient temperatures and sometimes in an inert atmosphere. Simple autosamplers inject each sample in sequence. Advanced models allow scheduling of out of sequence injections from selected vials according to a preset program. Low-cost autosamplers use a simple mechanism to pierce the septum cap and displace the sample solution by gas pressure or plunger action to the inlet port of an automated injection valve. This approach is only suitable for complete-fill loop injection and requires a wash cycle to avoid carryover in sequential injections. An alternative approach uses a motorized syringe to withdraw sample from the vial and transfer it to the loop of the injection valve. This approach can handle small sample volumes with minimum waste using either complete- or partial-fill loop injection. The most advanced autosamplers combine the functions of automated injection with aspects of robotic workstations. They can perform additional unsupervised routine operations such as dilutions, standard additions, derivatization, liquid or solid-phase extractions, and variable volume injections for calibration, etc.

5.3.2 Trace Enrichment (SPE-LC)

On-line coupling of a short column for solid-phase extraction (SPE) to a separation column with a switching valve as an interface provides a simple and reliable technique for automated sample preparation (isolation, concentration and matrix simplification) and liquid chromatographic separation (SPE-LC) [40-48]. On-line coupling of the two techniques is expected to result in greater accuracy and precision compared with manual methods by minimizing losses and contamination during sample handling, reduce solvent consumption and provide significant time and cost savings.

The choice of precolumn dimensions and sorbent properties is a compromise between the need for sufficient retention to accommodate the desired sample volume and efficient analyte desorption for transfer to the separation column without unacceptable loss in its separation power. Typical precolumns are 10-30 mm long with internal diameters of 1-3 mm for use with conventional diameter separation columns. These columns contain about 10-50 mg of sorbent with an average particle diameter in the 10 to 40 μm range. Typical sample volumes for SPE-LC are 1-10 ml for general applications and 10-1000 ml for environmental applications. Low-specificity sorbents (e.g. octadecylsiloxane-bonded silica and porous polymers) are used for general applications [42,49,50], and class-specific sorbents, particularly restricted access materials and immunosorbents, for selective extraction of target analytes [40,41,51-55]. Restricted access materials have gained acceptance for the analysis of drugs in biological fluids as well as polar pesticides in the presence of humic acid in surface waters. Direct injection of untreated plasma or serum, and to a lesser extent urine, samples onto reversed-phases columns is made difficult by the incompatibility of the sample matrix with the separation system. Sample proteins become adsorbed on the solid phase and denatured or precipitated by components of the mobile phase, shortening the useful lifetime of the analytical column. Use of a short precolumn serves the dual function of acting as a guard column as well as affecting a preseparation of the

analytes from the biological matrix. Restricted access sorbents have an internal surface of retentive bonded phase and an external surface designed to be weakly retentive of biopolymers. The macromolecules found in biological fluids are excluded by their size from exploring the pore volume and are eluted within the void volume of the precolumn avoiding any destructive accumulation on the sorbent. The smaller analyte molecules have access to the pore volume and are retained there until subsequently desorbed with an appropriate solvent on to the analytical column for separation. Restricted access sorbents are reasonably durable and long lifetimes have been obtained.

Breakthrough volume considerations are generally unimportant for sampling biological fluids, since only small sample volumes are required for adequate detection of target analytes but for environmental applications this is a critical parameter and dominates sorbent selection. High surface area porous polymers, and to a lesser extent carbon sorbents, are generally used for environmental applications [45,56-60]. Low-selectivity sorbents in conjunction with pH adjustment, if required, enable most analytes of low to moderate water solubility to be efficiently collected. Since the precolumns are short, pressure drops are low, and the sample solution can be pumped through the precolumn at high flow rates, typically 5-25 ml / min. Sampling times are thus short, even for sample volumes of 0.01-1 liter. Many contaminants at concentrations of 0.1-10 $\mu\text{g} / \text{l}$ can be determined routinely in surface waters using fully automated coupled-column systems with UV or mass spectrometric detection

The on-line coupling of precolumn and separation column is achieved using a single six-port automated switching valve with flow paths as indicated in Figure 5.6, or by using two automated valves for additional versatility in automating a wider range of sample processing steps. The control system has to provide for regeneration and conditioning of the precolumn or automated exchange of the precolumn for each sample application, loading of the sample, rinsing the precolumn to minimize matrix constituents and transfer of the analytes to the analytical column for separation. Usually the sample processing steps occur concurrently with the separation of the previous sample. All events are programmable and optimized by time and flow control.

For strongly retained analytes, backflushing the precolumn is often the best approach for solvent desorption. The desorption solvent is usually the same or a weaker solvent than the mobile phase used for the start of the separation. Since the analytes are distributed over a significant length of the precolumn their transfer to the separation column often requires a solvent volume that is too large and/or a solvent that is perhaps too strong to avoid additional band broadening compared with conventional injection techniques. The precolumn dimensions (short column of smaller internal diameter than the separation column), sorbent particle size (small particle diameter) and the retention capacity of the sorbent for the analytes (weak retention capacity) are optimized to assist in minimizing band broadening due to the transfer process. On the other hand for successful sampling the sorbent should have strong retention properties, the precolumn should be as large as practical and packed with coarse particles to assist in sample application when large volumes are used. The criteria for extraction and desorption are generally opposite and practical operating conditions are always a compromise.

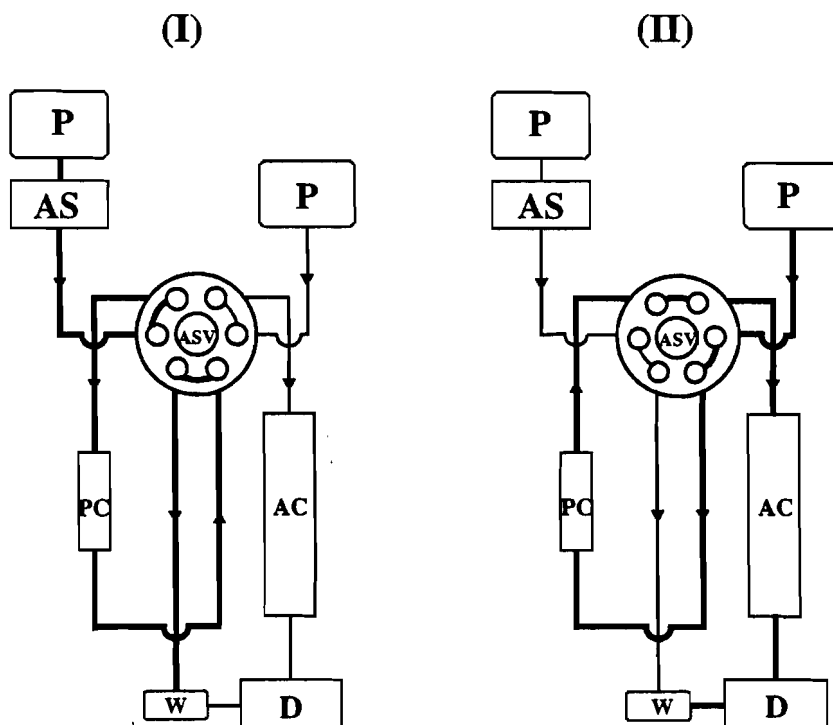


Figure 5.6. Arrangement for automated coupled-column switching in liquid chromatography. Position (I) is for sample application and fractionation and position (II) for transfer of extracted analytes and subsequent separation. P = pump, AS = autosampler, ASV = automated switching valve, PC = precolumn, AC = analytical column, D = detector and W = waste. (From ref. [41]; ©Elsevier).

In most cases preconcentration of analytes occurs with simultaneous matrix accumulation. Matrix simplification can be achieved through the sampling process, the use of a rinse solvent prior to analyte transfer, and selective transfer of front-, heart- or end-cuts of the desorbed sample extract. Dual precolumn systems allow solvent exchange and separate trapping of analytes with a wide range of breakthrough volumes. The first column of a dual precolumn system can be used to retain strongly sorbed matrix components that would contaminate the second precolumn or interfere in the separation of the transferred extract.

The volume of sample solution that can be processed without breakthrough on a precolumn depends on the dimensions of the precolumn, the retention properties of the sorbent, the sample solvent (usually water), and the flow rate used during sampling. The breakthrough volume can be measured experimentally or estimated from chromatographic data [45,61,62]. In the first approach a solution containing a low but detectable concentration of analyte is pumped at a constant flow rate through the precolumn, which is connected directly to the detector. The analyte response at the

precolumn exit is a breakthrough curve from which the breakthrough volume can be determined. Since the breakthrough volume is flow dependent the flow rate used to record the breakthrough curve should be similar to the flow rate used for sample application. The breakthrough volume can be estimated for aqueous samples using Eq. (5.2)

$$\log V_B = \log QV_M + \log (1 + k_w) \quad (5.2)$$

where V_B is the breakthrough volume, Q the contribution of kinetic factors to retention resulting from the small plate number for the precolumn, V_M the precolumn void volume and k_w the solute retention factor with water as the mobile phase. System extracolumn volumes can be significant for short precolumns translating into uncertain values for V_M and the plate number required for the calculation of Q . Retention factor measurements are subject to substantial errors whether determined directly with water as the mobile phase or by extrapolation from measurements made with binary mobile phases containing an organic solvent (section 4.3.1).

A number of interfaces have been described for coupling the coated fused-silica fibers used for sampling in solid-phase microextraction (SPME) to liquid chromatography [63]. For this purpose, the injection loop on a standard injection valve is replaced by a small-volume desorption chamber that allows insertion of the fiber into the chamber at atmospheric pressure. Desorption of the analytes at the operating pressure of the liquid chromatograph is performed by elution using either the mobile phase or a separate solvent, aided by heating, if necessary. This process is difficult to automate, can produce broad peaks for strongly sorbed compounds and is somewhat limited in sensitivity by the sample capacity of the fiber. A more favorable approach is represented by in-tube SPME [63-65]. In this case the extraction device is a length of fused-silica capillary column coated with a typical gas chromatography stationary phase or polymer such as poly(pyrrole). The capillary column is placed between the sample injection loop and injection needle of an autosampler with minimal changes to the normal configuration of the autosampler. For extraction the autosampler is programmed to draw the sample into the capillary from a vial and then eject the sample back into the same sample vial. The optimization parameters are the number of draw/eject cycles (typically 1-20), column length and internal diameter (typically 60 cm x 0.25 mm to give a sample volume of about 30 μ l), sample flow rate (100 μ l / min) and film thickness or active surface area of the sorbent. The analytes are desorbed from the extraction capillary with mobile phase or other solvent into the injection loop of the autosampler for injection onto the separation column. There is a need for improvements in sorbent technology to increase the extent or speed of extraction reached at equilibrium. With columns that currently exist and standard instrumentation analyte concentrations in the range 50-250 ng / ml with an extraction time of 5-15 minutes have been demonstrated. Matrix sorption or interferences may limit the approach for some samples.

5.4 GUARD AND SCAVENGER COLUMNS

A guard column is a short column placed between the injector and the separation column as either a separate device or an integral component of the column inlet fitting [66,67]. Its purpose is to extend the life of the separation column by protecting it from deterioration caused by sample contaminants and highly retained solutes, as well as from wear particles from the pump and injection valve. It also functions as a saturator column to prevent dissolution of the stationary phase in the separation column, although a scavenger column should be used for this purpose. Since it is located in the sample pathway, it must be packed with the same material and to the same packing density as the separation column otherwise unacceptable peak broadening in the separation may be observed. To maintain an adequate capacity for sample impurities the volume ratio of the guard column to that of the separation column should be in the range 1:15 to 1:25. Guard columns are meant to be sacrificed as sample and operating conditions dictate.

A scavenger column is a column of various sizes placed between the pump and the injector [68,69]. Its main function is to act as a saturator column for silica-based column packings when using aggressive mobile phases (e.g. pH > 8 in reversed-phase chromatography). Simultaneously it removes unwanted contaminants from the mobile phase and acts as a filter to protect the separation column from particle matter, pump-seal wear particles, etc. The column is not located in the sample pathway and need not be identical to the separation column in particle size or packing density. For reasons of economy, and to minimize the operating pressure, scavenger columns are usually packed with coarse particles but of a fairly narrow size range. For isocratic operation the size of the column is not too important, as long as it has sufficient capacity to perform its function. With gradient systems the scavenger column must be small to minimize the gradient delay volume. Scavenger columns are optional components used only when dictated by the characteristics of the mobile phase.

5.5 COLUMN TEMPERATURE CONTROL

Most separations in liquid chromatography are performed at room temperature for convenience and because ambient temperatures provide reasonable column efficiency for low molecular mass solutes. Elevated temperatures are commonly used in ion-exchange chromatography to improve mass transfer kinetics and in size-exclusion chromatography to provide adequate solubility for polymers in useful mobile phases. Wider interest in temperature control and high-temperature separations in general results from improved precision of retention measurements (section 1.1.1), greater column efficiency (section 1.5.2), the use of temperature as a variable for method development (section 4.4.4), and shorter separation times due to the more favorable use of the column inlet pressure [70,71].

Column thermostats are required to maintain a constant temperature ($\pm 0.1^\circ\text{C}$) in both time and space. Differences in temperature between the mobile phase entering

the column and the column oven can result in both axial and radial temperature gradients within the column. Frictional heat generated by the movement of the mobile phase through the column is a further potential source of radial temperature gradients (section 1.5.1). Radial temperature gradients result in retention and viscosity variations in the column manifested by reduced column efficiency and possibly peak asymmetry and sometimes peak splitting. When the incoming mobile phase is at a lower temperature than the column thermostat the temperature at the column wall exceeds that at the column center. As the mobile phase moves through the column, the temperature at the column center gradually approaches that at the wall. A temperature difference of about 5°C or more between the incoming mobile phase and the column thermostat is sufficient for significant band broadening for columns of conventional diameters. Differences between the column temperature and set point temperature result in changes in retention factors and selectivity that affect method transferability between instruments.

Column thermostats from different manufacturers vary widely in their design and the presence or effectiveness of methods to preheat the mobile phase. The thermostat is usually an air circulating oven, block heater or circulating liquid (usually water) bath. Peltier block heaters, for example, can be used to set temperatures between 5 and 90°C. Preheating the mobile phase is achieved by positioning a coil of stainless steel capillary tubing between the injector and column. The capillary tube is housed in the column thermostat with the injection valve outside the thermostat unless the injection valve requires thermostating to keep the sample in solution. The required length and internal diameter of the preheater coil for specific applications depends on the temperature difference between the incoming mobile phase and the column thermostat, the mobile phase flow rate and the heat transfer properties of the thermostat and capillary tube. The required dimensions of suitable preheating coils are amenable to calculation [70,71]. The preheating coil represents additional extracolumn volume and should be no longer or wider than necessary. A capillary tube of 25 cm x 0.02 mm internal diameter has a volume of about 10 µl and is well tolerated by conventional diameter columns operated at normal flow rates. This should suffice for most normal applications where temperature differences are unlikely to be large (10-40°C). For high temperature and pressurized hot water liquid chromatography larger preheating coils are required.

Columns of conventional diameters have a relatively high heat capacity and are unsuitable for use in temperature programmed separations. Packed capillary columns, on the other hand, respond rapidly to changes in temperature and are suitable for temperature programmed separations even at high program rates [72-75]. Temperature programmed separations have been used as a complement to gradient elution separations, although the normal elution range that is possible by varying temperature is not as great as that available by varying mobile phase composition. In addition, temperature programmed separations have been used for the structural elucidation of polymers and identification of polymer additives using relatively long packed capillary columns and non-aqueous mobile phases. In most cases gas chromatography-type ovens were used for temperature control and a narrow bore fused-

silica capillary restrictor between the column and detector or a backpressure regulator after the detector to maintain the mobile phase in the liquid state.

5.6 COUPLED-COLUMN SYSTEMS

Coupled column (or column switching) techniques encompasses a variety of techniques used for sample separation, cleanup and trace enrichment [76-81]. Applications involving sample preparation are discussed in section 5.3.2. The most important separation applications are multidimensional liquid chromatography (section 5.6.1) and comprehensive two-dimensional liquid chromatography (section 5.6.2). A characteristic feature of these methods is the use of two or more columns for the separation with automatic switching of fractions between columns by a valve interface. These techniques require only minor modification to existing equipment, and of equal importance, enable the sample preparation and separation procedures to be completely automated.

5.6.1 Multidimensional Liquid Chromatography

A basic system for multidimensional liquid chromatography requires addition of a manual or automated multiport switching valve and a second column to a standard liquid chromatograph. The switching valve is located between the two columns and manages the mobile phase flow direction at the exit of the first column and entrance to the second column, including the transfer of sample components between columns. More advanced systems may use several switching valves to allow full automation of several functions, such as column conditioning, sample loading, matrix simplification, sample transfer between columns using backflushing, foreflushing or heartcutting techniques, elution from either column, etc [77-83]. In complex chromatograms the precise location of the area of the chromatogram to be switched is important to minimize co-transfer of sample components that may interfere in the quantification of relevant peaks. An in-line detector located between the first column and the switching valve can be used to locate the region of the chromatogram to be switched for each sample. Alternatively, a sequential timer can be used to automatically switch the peaks of interest. Interval timers, however, are unable to account for variability in analyte elution volumes from the first column.

Switching valves are similar in construction and operation to rotary injection valves (section 5.3.1). The switching valve must be capable of high-pressure operation without deterioration, and provide a low-dead volume flow path so as not to significantly broaden the peaks that pass through it. The valve may also contain a fixed-volume loop for trapping the transferred peaks prior to injection onto the second column, or it may simply be used as a switch to divert flow between columns.

Two general considerations are important for selecting the columns to be coupled. If a significant improvement in resolution is to be obtained then the retention mechanisms

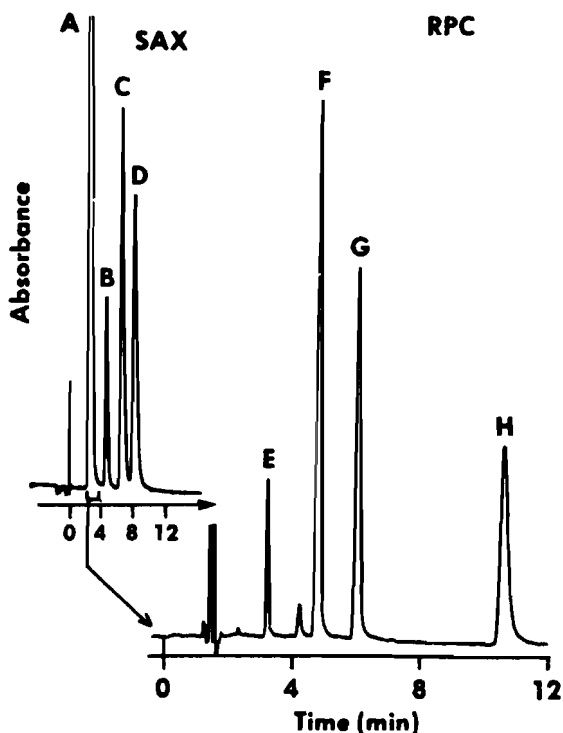


Figure 5.7. Separation of deoxyribonucleosides and their 5'-monophosphate esters by multidimensional liquid chromatography on a strong cation-exchange column (column one) and a reversed-phase column. The unseparated nucleosides, peak A, on the ion-exchange column were switched to the reversed-phase column for separation. Peak identification: A = nucleosides, B = d-CMP, C = d-AMP, D = d-GMP, E = d-CYD, F = d-URD, G = THD, and H = d-ADO. (From ref. [84]; ©Preston Publications, Inc.).

for the two columns must be complementary. In addition, the mobile phase flow rate and solvent strength must be compatible with transfer (injection) of the sample components onto the second column. The flow rate for the first column must be less than the flow rate for the second column. The mobile phase for the first column must be a weak solvent with respect to the separation mechanism on the second column and must be miscible with the mobile phase for the second column. In this way the bandwidth of the transferred zone at the start of chromatography on the second column is minimized by reconcentration at the column inlet. Because of these practical requirements not all column types are compatible. Typical examples of compatible systems are size-exclusion with normal, reversed-phase and ion-exchange chromatography; ion-exchange with reversed-phase chromatography; and polar bonded phase with normal-phase chromatography. An example of a multidimensional separation on a strong cation-exchange column and a reversed-phase column is shown in Figure 5.7 [84]. The neutral deoxyribonucleosides are switched as a single peak for separation on

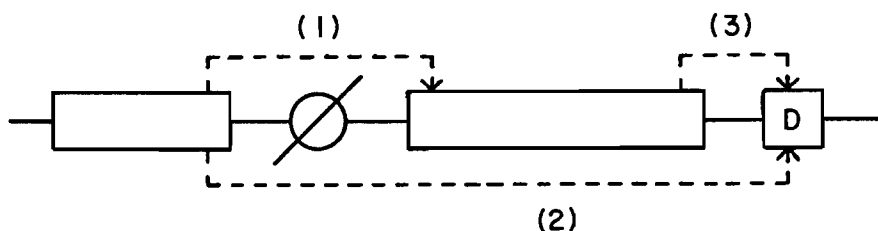


Figure 5.8. Schematic diagram of a two-column system for the separation of a sample containing components with a wide range of retention factors.

the reversed-phase column while the ion-exchange column resolves the phosphate-containing analogs.

Multidimensional liquid chromatography provides an alternative approach to gradient elution for tackling the general elution problem. Two columns containing the same stationary phase but of different lengths or two columns of similar length but of different selectivity are used, Figure 5.8. The first column is short (or of low selectivity) and is used to separate the later eluting components of the chromatogram. The components of low retention are switched to the second column (1) and remain there while the most retained components are separated on the first column (2). When these components have been separated, the least retained components are then separated on the longer (or more selective) second column (3). Compared to the retention order in the normal chromatogram, the retention order in the multidimensional chromatogram is reversed; the most retained components are at the front of the chromatogram while the least retained are at the back. The total separation time is reduced and, as the peaks elute in a smaller volume of mobile phase, they are easier to detect. Isocratic operation simplifies the instrumentation required, permits the use of gradient incompatible detectors, and eliminates the problem of column equilibration required in gradient elution separations.

Multidimensional liquid chromatography is preferred over gradient elution when a large plate number is required for a separation and for the analysis of complex mixtures which contain only a few adjacent components of interest. For these problems, single-column techniques are inherently inefficient, since only a small fraction of the column is actually in use at any given time. The multidimensional separation methods provide optimum efficiency for the separation of the components of interest while simultaneously minimizing the time spent in separating sample components of no particular interest.

5.6.2 Comprehensive Two-Dimensional Liquid Chromatography

Multidimensional techniques are used to enhance the resolution of selected regions of a chromatogram by isolation and transfer of a group of neighboring components to a second column for further separation (section 5.6.1). A multidimensional technique is considered comprehensive when the whole first dimension separation, and not just a selected region of this separation, is subjected to further separation in a second

dimension (chromatographic system) without diminishing the fidelity of the first dimension separation [85,86]. The results from a comprehensive two-dimensional separation are usually represented as a contour plot with each chromatographic separation along an axis. In common with multidimensional chromatography, if a significant increase in the peak capacity is to be achieved then the separation mechanisms for the coupled separation systems must be complementary. In addition, for ease of automation the mobile phase for the first dimension separation must be a weak solvent with respect to the separation mechanism employed for the second dimension otherwise the volume of the transferred sample would be prohibitively restricted. A comprehensive system, however, differs from a multidimensional system in the sampling frequency of the first dimension separation. To preserve the resolution achieved in the first dimension separation requires that each peak is sampled at least three (and preferably more) times across the peak width by the second dimension [87]. Resolution in the second dimension is not affected by undersampling the first dimension, but the overall separation is diminished because of the loss of resolution along the first dimension axis. Simple tandem coupling of two columns is not comprehensive because components separated on the first column may remerge on the second column.

The required sampling frequency dictates that the second dimension separation should be as fast as possible while providing adequate resolution and the first dimension separation should be slowed down to accommodate the sampling frequency and second dimension separation time. The total separation time is the product of the second dimension separation time and the total number of fractions injected into the second dimension. Thus, the separation time of the second dimension separation is a major factor in determining the total separation time of comprehensive two-dimensional separations. When more than one separation dimension is utilized in a sequential coupled column mode, a larger dilution of the original injection concentration occurs with loss of sample detectability [88]. The column dilution factors and split ratios used to compute limits of detection are multiplicative per dimension. In critical cases information may be lost when a fraction transferred from the first dimension falls below the detection threshold after separation in the second dimension.

The comprehensive features of a two-dimensional system are achieved through sampling the first dimensional separation continuously at a sufficiently high frequency. Off-line collection of sequential portions of effluent from the first column and injecting them into the second column one at a time or using stopped-flow techniques is too time consuming and impractical for general use [89,90]. A popular interface employs an automated eight-port switching valve with two identical sample loops [87,91-93]. Effluent from the first column alternately fills one loop at a time. While one loop is being filled the contents of the other loop is injected and separated on the second column. A computer is used for time sequencing of the valve positions. A different approach uses an automated ten-port switching valve as an interface between the first dimension separation column and two identical columns in parallel for the second dimension separation [94-96]. One of the second dimension columns is being loaded with sample

from the first dimension column while the second column is separating the previous fraction. The loading time and separation time for the second dimension columns are identical. Fast rotation of the switching valve ensures that effluent from the first column is loaded continuously on either of the two columns in sequence with separation of the previous fraction on the other column.

Comprehensive multidimensional liquid chromatography is a relatively new development and has yet to develop a diverse application base. For the time being applications are dominated by the separation of proteins and synthetic polymers. For proteins the first dimension separations are usually based on ion exchange and the second dimension separations on reversed-phase liquid chromatography. Gradient elution was often used for both separation modes with a separation time less than 2 minutes for the second dimension separation and from 30 minutes to several hours for the first dimension separation. Current trends include the use of non-porous particles and perfusive stationary phases for the second dimension separation to reduce the total separation time and wider internal diameter columns in place of packed capillary columns to simplify interface construction and instrument operation and to allow the loading of larger sample sizes.

Two-dimensional comprehensive separations of proteins are also possible by coupling reversed-phase or size-exclusion liquid chromatography and capillary electrophoresis [97-100]. The low flow rates characteristic of capillary electrophoresis requires an interface design capable of transferring small volumes while minimizing extracolumn band broadening, Figure 5.9. The transverse flow-gating interface uses a transverse flow of buffer through the narrow space between capillary columns to prevent transfer of column eluent to the electrophoresis capillary until injection is required. For injection the transverse buffer flow is stopped allowing the eluent to flow across the gap to the electrophoresis capillary. By applying an appropriate voltage for the desired time an electrokinetic injection of the column effluent onto the electrophoresis capillary is obtained. After injection, the appropriate separation voltage is re-established for the electrophoresis capillary and the transverse buffer flow reinstated to direct the column effluent to waste. The critical operating parameters are the distance between capillaries, the transverse buffer flow rate, the injection time and voltage, and the ramp rate used to adjust the electrophoresis voltages between the injection and separation values. Overlapping injection (injection at half of the separation time so that two sample injections are present in the electrophoresis capillary at any time) allows the sampling frequency of the first dimension to be increased.

5.7 DETECTORS

Separations in liquid chromatography occur in a dynamic manner and, therefore, require detection systems that work on-line and produce an instantaneous record of column events. A prototypic detector for analytical liquid chromatography must have good sensitivity to deal with low analyte concentrations, a small detector volume to minimize

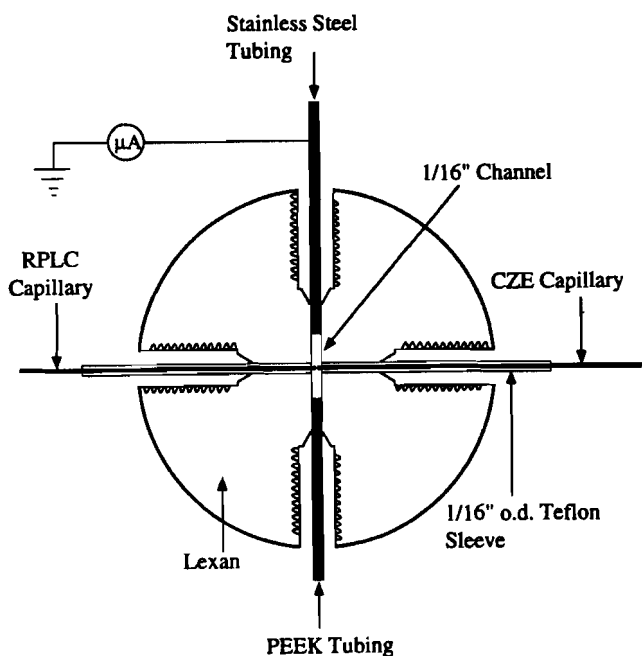


Figure 5.9. Schematic diagram of the transverse flow gating interface for coupling capillary liquid chromatography and capillary electrophoresis in comprehensive two-dimensional separations. The interface is constructed from clear Lexan to allow visual positioning of the two capillary columns. (From ref. [100]; ©American Chemical Society).

extracolumn dispersion, and a fast response compatible with the rapidly changing analyte concentrations. Detectors in common use belong to one of two classes: bulk property or solute property detectors [1-3,5,101-106]. Bulk property detectors, such as the refractive index detector, measure the difference in some physical property of the solute in the mobile phase compared to the mobile phase alone. These detectors are fairly universal in application but generally have low sensitivity and a limited dynamic range arising from the fact that the detector signal depends on the difference in properties between the solute and the mobile phase and not on solute properties alone. Thus bulk property detectors are usually adversely affected by small changes in the mobile phase composition and temperature, which usually precludes the use of flow, temperature or composition programming.

Solute property detectors, such as spectrophotometric detectors, respond to a physical or chemical property characteristic of the solute, which is ideally independent of the mobile phase. Although this criterion is rarely met in practice, the signal discrimination is usually sufficient to permit operation with solvent changes (e.g. flow and composition programming) and to provide high sensitivity with a wide linear response range. For the analysis of complex mixtures with the modest plate numbers characteristic of

liquid chromatography detector selectivity can be as important as column selectivity in obtaining an acceptable result. In addition, the higher sensitivity of solute property detectors is not only important for trace analysis but also for compatibility with the miniaturized detector volumes associated with small bore and capillary columns. For these reasons it is not surprising that solute property detectors are more widely used for general applications than bulk property detectors, but since they fail to provide a universal detection mode, bulk property detectors remain essential for some applications.

5.7.1 Spectrophotometric

The most important of the spectrophotometric detectors are the UV-visible absorption detector, the fluorescence detector and the chemiluminescence detector [5,101-108]. The UV-visible absorption detector is the most widely used detector in liquid chromatography. Since most organic compounds absorb to some extent in the UV, these detectors are reasonably universal in application. The detector response, however, depends on how strongly the sample absorbs light at a particular wavelength and the availability of a transparent mobile phase for the separation corresponding to the wavelength of maximum absorption. Organic compounds absorb most strongly in the wavelength range 180-210 nm, which is also the range in which most solvents have strong absorption bands, making detection difficult in this region. Longer wavelengths are typically used in practice reducing both detector sensitivity and the possibility of a universal response. The operation of absorption detectors is based on the measurement of the absorption of monochromatic light in accordance with the well-known Beer-Lambert law. Most detectors provide a signal in absorbance units which is linearly related to sample concentration over a range of 10^4 to 10^5 and detection limits, for the most favorable cases, of about 0.1-1.0 ng (about 10^{-8} M in concentration terms).

Fluorescence detection is inherently more sensitive than absorption detection but has a restricted application range. Only a small fraction of all organic compounds that absorb light are naturally fluorescent, and then only a still smaller fraction are strongly fluorescent. The molecular requirements for fluorescence cannot be clearly defined, but many strongly fluorescent compounds contain rigid, planar, conjugated systems. Further selectivity in the detection process arises from the use of two different wavelengths in the measurement process, an excitation wavelength and an emission wavelength. In contrast to absorption measurements, the emitted radiation is measured against a dark background due to optical separation of excitation (absorption) and emission (signal) processes, permitting the use of higher signal amplification. Under favorable circumstances detection limits of 1-10 pg with a linear range of about 1000 are possible. Laser-based fluorescence detectors are capable of still lower mass detection limits and enable very small detector volumes to be used to preserve efficiency in packed capillary column separations [109-111].

Chemiluminescence differs from fluorescence in the mechanism used to generate the excited state species responsible for the emission signal [104,112]. In the case of

fluorescence this occurs by the absorption of light while for chemiluminescence an exothermic chemical reaction performs this function. The absence of an excitation source results in a lower emission background signal and lower detection limits in favorable circumstances. A typical arrangement for chemiluminescence employs a postcolumn reactor to mix the column effluent with chemical reagents responsible for analyte excitation.

5.7.1.1 Absorption Detectors

Absorption detectors vary in their ability to record single or multiple wavelength chromatograms and the possibility of providing full spectra for each peak in the chromatogram [1,105-107]. Fixed single-wavelength detectors employing atomic vapor sources, a simple filter, and a photodiode detector are the simplest, and among the most sensitive, of the absorption detectors. Typical atomic vapor sources include mercury with emission lines at 254, 313 and 365 nm (among others), cadmium at 229 and 326 nm and zinc at 308 nm [113,114]. The source emission lines are narrow and well separated allowing detector construction without a monochromator, reducing the cost of the detector. The number of wavelengths available for detection can be increased by using a phosphor screen to re-emit the source light at a longer wavelength, for example, using the mercury atomic vapor lamp the emission line at 254 nm can be re-emitted at 280 nm. By using a highly regulated source, dual beam operation and photodiode detectors, a high signal-to-noise ratio can be achieved. The main limitation of this design is that the sample must have some absorption at the source wavelength to be detected and any possibility of optimizing selectivity is restricted to the few source lines available. These problems are overcome using continuously variable wavelength detectors or multichannel detectors.

The variable wavelength detector utilizes a continuum source combined with a rapid scanning monochromator for wavelength selection. Programmed wavelength changes during a separation and recording of complete absorption spectra for selected peaks are possible with some detector designs. High energy sources, typically a deuterium discharge lamp for the range 190-350 nm and a tungsten lamp for the range 350-800 nm, and relatively low optical resolution (i.e. moderate bandpass monochromators) to maximize energy throughput while maintaining an acceptable linear response range are generally used. An example of a variable wavelength detector is shown in Figure 5.10A. Dual beam operation is almost universal to compensate for short-term fluctuations in the source intensity and electronics. Source fluctuations arise from two principal causes [113]. Eddies developed around the lamp envelope as a result of the heat generated by the lamp and the consequent variation of the refractive index of the surrounding air leads to fluctuations in the amount of light falling on the detector cell. An air circulation fan is often used to improve the dissipation of heat from the source housing. The lamp arc is not homogeneous over its length, or over time, and variations in spectral output and intensity can occur. In practice, electronic noise and lamp noise is almost negligible when using dual beam operation compared to the noise originating at the detector flow cell, which effectively limits sensitivity with contemporary detectors.

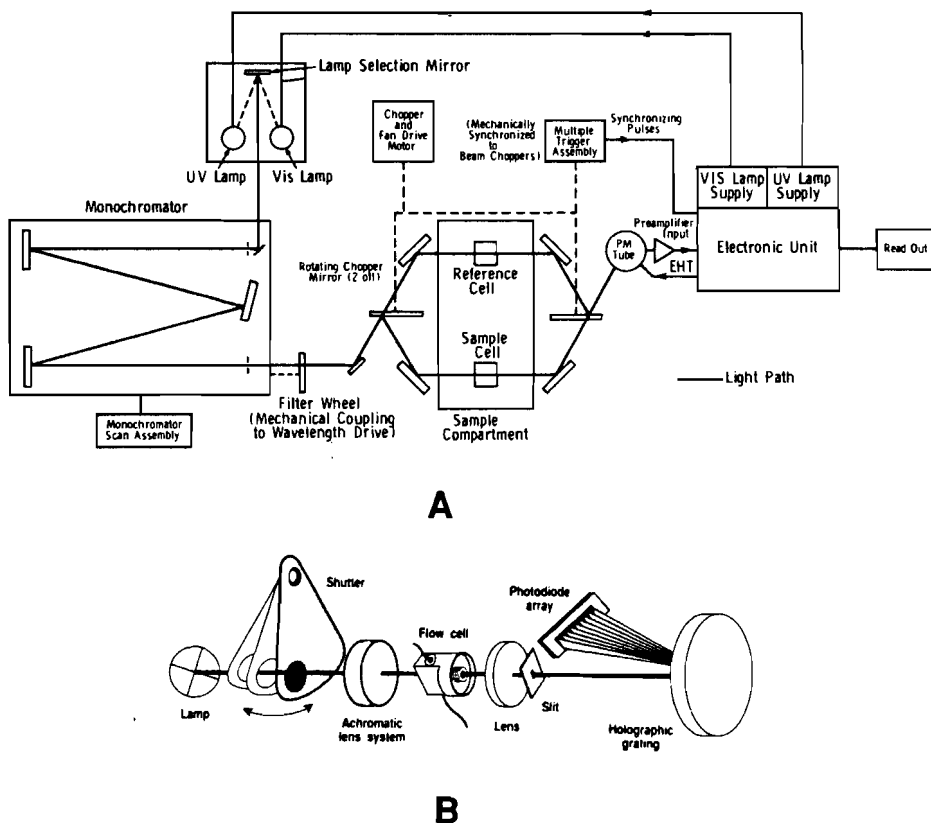


Figure 5.10. Schematic diagram of a variable wavelength dual beam absorption detector (A) and a diode array detector employing reverse optics (B).

Diode array detectors employ a multichannel approach to data collection in which an array of photodiodes is placed at the focal plane of a polychromator, Figure 5.10B. All wavelengths from the source are focused through the flow cell and subsequently dispersed by the grating for simultaneous measurement. Two unusual features of this arrangement are that the sample is illuminated with a broad wavelength source instead of monochromatic light and the optical system contains no moving parts. In this configuration the photodiode array acts as a multichannel radiation sensor, storage device, and readout system. The diode array consists of several hundred photosensitive diodes (e.g. 1024, 512, 256, etc.) generally configured in a linear pattern on a silicon wafer. Each photodiode is connected in parallel to a storage capacitor. The photocurrent generated by light striking individual photodiodes discharges their respective capacitors. Each photodiode is successively interrogated by a digital shift register and the integrated light intensity at each diode recorded. The entire readout process is repeated sequentially

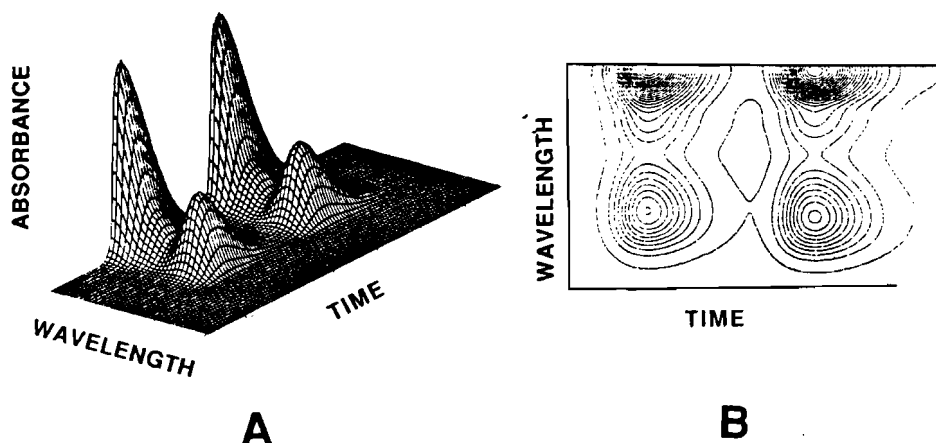


Figure 5.11. Examples of the display options for chromatographic and spectroscopic data obtained from a diode array detector. A three-dimensional isometric plot of absorbance, wavelength and time is shown in A. The same data plotted as a contour diagram is shown in B. (From ref. [119]; ©Elsevier).

every few microseconds. A computer (or series of microprocessors) is required to manage the data acquisition tasks and to process the information. Diode bunching, in which the output from a group of adjacent diodes is electronically summed or averaged to produce a single output is used to improve the signal-to-noise performance. Practically, this is accomplished by defining a central wavelength for detection and an associated effective bandwidth. These parameters, though, must be set carefully to avoid non-linearity in the calibration plots. Certain diodes (reference wavelengths) can be designated to reduce baseline disturbances due to source instability and refractive index effects in the flow cell. This approach is used to reduce baseline drift in gradient elution separations.

Differences in diode array detectors can be found in how they acquire and manipulate data, either in real time or in post run calculations, and in display procedures [107,115-118]. Full spectra are recorded continuously at microsecond time intervals and stored as raw data, or in an abbreviated form. Data is stored as a matrix where the rows are spectra measured at different time intervals and the columns are chromatograms measured at different wavelengths. The data matrix can be interrogated in several ways and at a later date, should the need arise. A common format for viewing the total data matrix is a three-dimensional isometric plot with axes of absorbance, wavelength and time as shown in Figure 5.11A [119]. Hidden line removal and false color can be used to minimize loss of information for small peaks obscured by earlier eluting more intense peaks. A contour plot provides an alternative method of data display, Figure 5.11B, where concentric isoabsorbance lines are plotted in the wavelength and time plane. The contour plot diagram facilitates the identification of optimum wavelengths for detection of each component and the identification of coeluting impurities as distorted contour profiles.

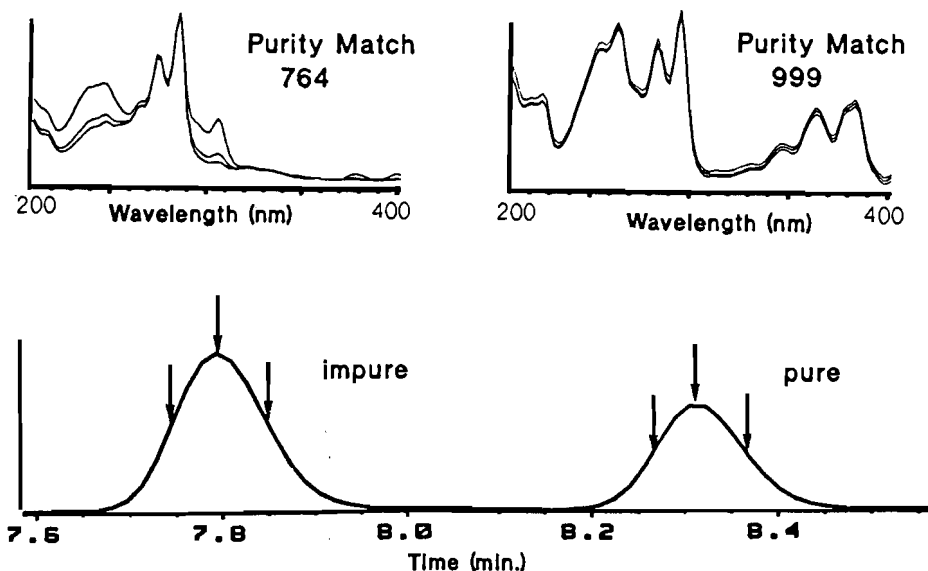


Figure 5.12. Example of peak purity determination by matching spectra recorded at different peak positions. (From ref. [107]; ©Marcel Dekker).

Diode array detectors are also used for peak purity and homogeneity analysis and peak identification. One common method of peak purity analysis is based on the use of absorbance ratios [119]. The computation of the absorbance ratio at two wavelengths for all points across the peak profile is a square-wave function for a single-component peak. Coeluting impurities, which absorb at least one of the selected wavelengths, will distort this function. The selection of detection wavelengths and the absorption threshold value can dramatically affect the utility of this technique. A superior approach is the comparison of the various spectra recorded during the elution of the peak to either a reference spectra or to each other. If an impurity has roughly the same peak shape as the main component but elutes at a slightly different retention time, then the level of the impurity will not be constant across the peak. Differences are expected to be greatest at the beginning and end of the main peak but the extreme regions of the main peak are most affected by noise and therefore less useful. A more satisfactory solution is the comparison of spectra recorded on the up slope, apex and down slope of the main peak, Figure 5.12 [107]. Irrespective of the actual way in which spectra are compared, an impurity is identified as a deviation in the match factor from the ideal value for identical spectra by an amount greater than can be attributed to noise. Even if the spectra are identical a peak can still be impure due to any of the following reasons: the impurity is present at a low concentration and therefore not detectable; the impurity has the same or very similar spectrum to the main component; or the impurity has the same peak profile and elutes at the same time as the main peak.

For overlapping peaks the data matrix contains linear combinations of the pure spectra of the overlapping components in its rows, and combinations of the pure elution profiles in its columns. Multivariate analysis of the data matrix may allow extraction of useful information from either the rows or columns of the matrix, or an edited form of the data matrix [107,116-118]. Factor analysis approaches or partial least-squares analysis can provide information on whether a given spectrum (known compound) or several known compounds are present in a peak. Principal component analysis and factor analysis can be used to estimate the maximum number of probable (unknown) components in a peak cluster. Deconvolution or iterative target factor analysis can then be used to estimate the relative concentration of each component with known spectra in a peak cluster.

Peak identification is based on the comparison of normalized spectra representative for the peak with spectra of one or several standard compounds run in the same separation system and stored in a spectral library [107,116]. This approach is less powerful than for mass or infrared spectral searches due to the rather broad and featureless bands that typify absorption spectra. Absorption spectra of similar compounds and compounds with a chromophore well separated from the variation in molecular structure are often virtually identical. Also, spectral changes dependent on the experimental conditions (pH, mobile phase composition, temperature, etc.) occur frequently. For this reason user prepared local libraries tend to predominate over general libraries, in contrast to common practices in infrared and mass spectral searches. A favorable spectral match for an absorption spectrum by itself is not acceptable for absolute identification.

Spectral matching is the process used to establish the similarity of compared spectra as a single number. Most matching procedures employ a point by point comparison of the two spectra in digital format to establish the presence or absence of significant differences. Individual matching procedures differ primarily in the methods used for spectral normalization and subsequently in the way spectral differences between spectra are converted to a single number. One common approach, for example, views each spectrum as a vector in multidimensional space and uses the angle between vectors (spectra) as a measure of their similarity. Library searches are usually performed as a forward or reverse search. In forward searches an attempt is made to identify each compound in an unknown sample from a large library of standard compounds. In a reverse search a limited library of standard compounds, all of which are likely to be present in the sample type, is searched against the unknown spectra in the current separation. Library searches can be combined with retention information to improve the certainty of identification. This is done by either weighting the match factor and retention similarity or by using the retention time to establish a retention window to preselect candidate spectra for comparison to the unknown spectra. Spectral quality is often an issue when matrix interference or noise precludes accurate spectral recording.

Careful consideration must be given to the design of the detector flow cell as it forms an integral part of both the chromatographic and optical systems. A compromise between the need to miniaturize the cell volume to reduce extracolumn band broadening

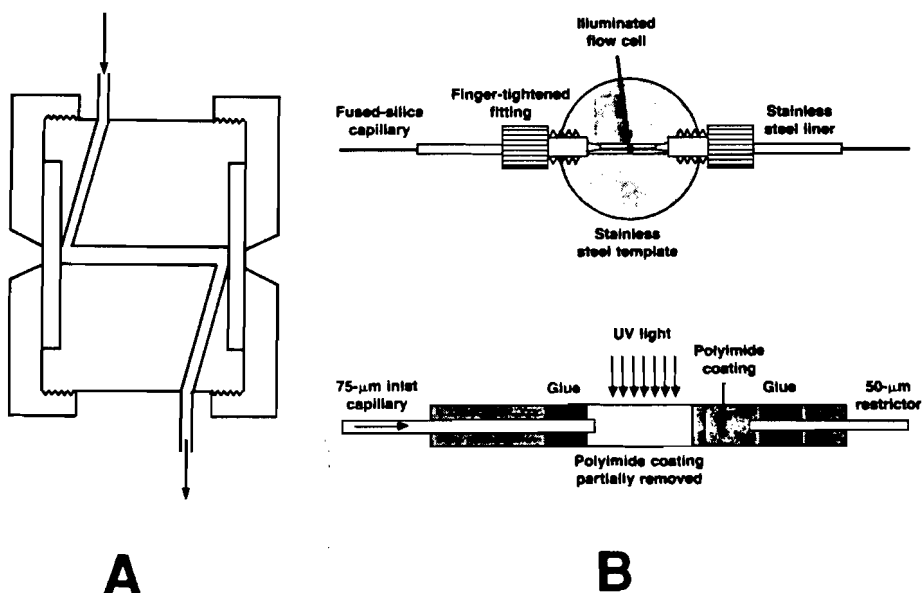


Figure 5.13. Representative flow cells used for absorption detection. A, Z-cell for conventional packed column applications and B, a low-volume fiber optic cell (volume = 4 nL and path length 0.15 mm) for use with packed capillary columns.

and the desire to employ long path lengths to increase sample detectability must be made. For packed columns of 4–8 mm internal diameter flow cells of the H-cell, Z-cell (Figure 5.13), or tapered cell configuration with a cell volume of about 5–10 μL and a path length of 1 cm, are commonly used [101,120]. The Z-cell design is used to minimize stagnant flow regions in the cell and to reduce peak tailing. Typically, the flow is confined by two quartz windows held in place by caps screwed onto the cell body at either end of the tubular cell cavity. The tapered cell has a conical cavity with a narrower aperture at the entrance than at the exit. The purpose of the tapered cavity is to reduce the amount of refracted light lost to the cell walls. Miniaturization of the same cell designs for use with small bore columns is possible by reducing the path length to 3 to 5 mm, providing cell volumes of about 0.2 to 2 μL . In order to maintain sensitivity the cell diameter should be reduced while maintaining the longest practical path length dictated by the cell volume requirements. Ultimately, however, reducing the cell diameter decreases the light throughput and increases background noise and susceptibility to refractive index changes. Commercially available flow cells suitable for absorption detection with small-bore columns have internal diameters of about 0.5 mm and may be internally coated with a reflective metallic layer to further reduce signal fluctuations caused by refractive index changes in the column eluent.

For absorption detection using packed capillary columns, detector volumes must be reduced to the nL range [109,114,121–124]. The simplest flow cells are prepared by

removing a short length of the polyimide protective coating from a fused-silica capillary, just beyond the retaining frit for the column packing. The cell volume and path length depends on the internal diameter of the fused-silica capillary. The detector volume is also defined by the slit width in the orthogonal direction to the capillary diameter, which cannot be made too small, otherwise there will be very little light transmitted through the capillary. Cell volumes from about 3 to 200 nl are easily created in this way. A fixed fused-silica capillary configured into a U- or Z-shape mounted in a holder can be more convenient in practice, since different columns can be attached using a short piece of PEEK tubing without changing the optical alignment for the cell. Also, the cell path length can be made wider than the internal diameter of the separation capillary to increase sample detectability at the possible expense of additional band broadening due to the increase in capillary diameters. Axial illumination achieved by coupling the source to a fiber optic positioned at the exit of the fused-silica capillary has also been explored to increase light throughput and the cell path length. The small size and cylindrical walls of capillary cells present problems in focusing the source energy into capillary cells. If a significant portion of the light passes through the cell wall, but not the fluid sample stream (as can easily be the case, since the column wall is typically thicker than the internal diameter of the capillary), the linear response range for the detector will be significantly reduced. Extreme sensitivity to refractive index perturbations is also common for all small-volume cell designs. A novel small-volume flow cell constructed from a cross fitting and two optical fibers is shown in Figure 5.13 [107]. The cell has no windows and can be located away from the source and photosensor. The exit capillary is narrower than the column internal diameter to maintain sufficient backpressure to avoid outgassing of the mobile phase. This cell has a volume of about 4 nl and a path length of 0.15 mm.

The principal source of background noise in modern absorption detectors is attributed to the inhomogeneous changes in refractive index of the eluent flowing through the cell [120,124-128]. This arises from temperature gradients, incomplete mixing of the mobile phase, flow perturbations, and turbulence. These effects cause some of the incident light, which would normally pass directly through the cell, to strike the cell wall instead, and is lost. The photosensor cannot distinguish light lost by refraction to the cell walls from light absorbed by the sample. The continuous variation of the refracted light contribution to the detector signal constitutes the major portion of the noise signal observed when the detector is operated at high sensitivity. Employing an optical design with aperture and field stops external to the cell is generally used to minimize the contribution from variations in the refraction of the flow cell.

5.7.1.2 Fluorescence Detectors

Fluorescence detectors differ mainly in the choice of excitation source and the method used to isolate the excitation and emission wavelengths [101-106,108,129,130]. Since the signal intensity is directly proportional to the source intensity, high-energy line (mercury) or continuous (deuterium or xenon) arc sources are used. The mercury source produces line spectra (254, 313 and 365 nm) which can be isolated with

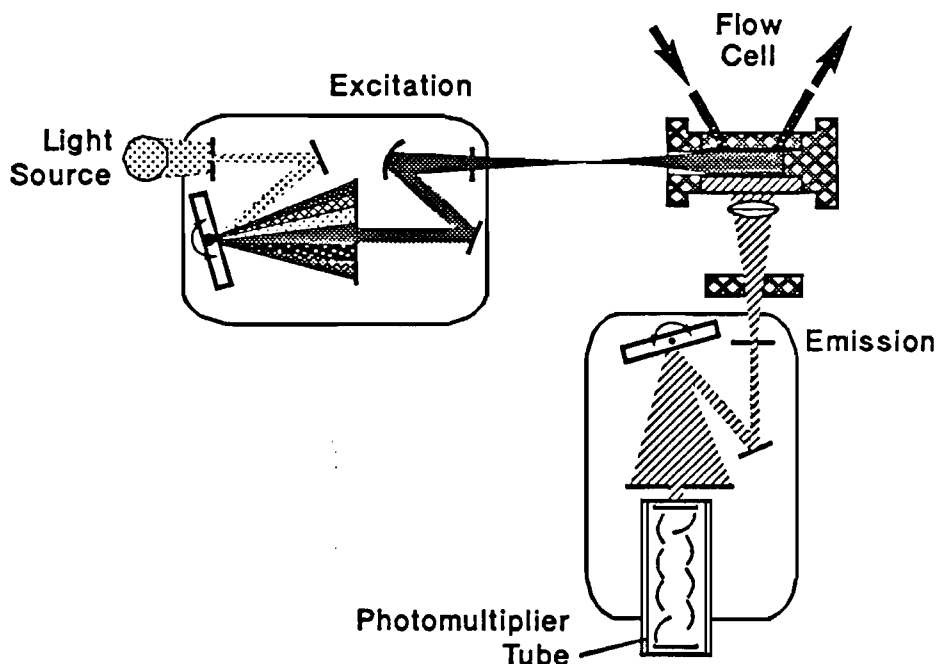


Figure 5.14. Schematic diagram of a fluorescence detector with rapid scanning monochromators for programmable selection of excitation and emission wavelengths.

a simple filter. However, the available emission lines may not overlap with the maximum excitation wavelength of the sample diminishing the detector response. Deuterium (190-400 nm) and xenon (200-850 nm) arc sources are used with a monochromator for continuously variable selection of the excitation wavelength, Figure 5.14. Fluorescence emission occurs in all directions and a hemispherical mirror can be used to maximize the fluorescence emission signal collected with an end-on photomultiplier detector. Emission wavelength isolation is performed using either filters or a monochromator. Filters are low cost and simple to use, however, there is generally a reduction in selectivity relative to wavelength selection using a monochromator because filters usually pass a wider range of wavelengths. Many modern detectors can be programmed to change either or both excitation and emission wavelengths during a separation to maintain optimum selectivity and sensitivity throughout the chromatogram, Figure 5.15. Some detectors using rapid scanning monochromators or a polychromator and an intensified diode array detector can record emission spectra at a fixed excitation wavelength as a function of retention time [108,130-133]. Less commonly rapid scanning monochromators or polychromators with diode array or charged coupled detectors have been used for the simultaneous recording of excitation and emission spectra as a function of retention time [119,129].

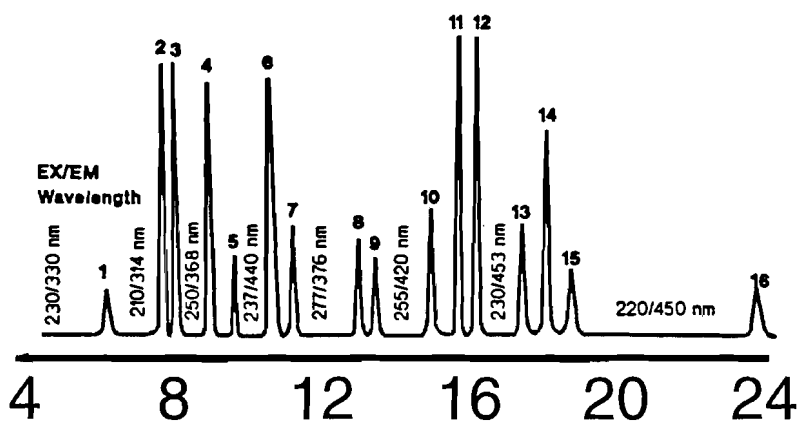


Figure 5.15. Reversed-phase gradient elution separation of a mixture of polycyclic aromatic hydrocarbons using time programmed fluorescence detection. Compounds: 1 = naphthalene; 2 = acenaphthene; 3 = fluorene; 4 = phenanthrene; 5 = anthracene; 6 = fluoranthene; 7 = pyrene; 8 = benz(a)anthracene; 9 = chrysene; 10 = benzo(b)fluoranthene; 11 = benzo(k)fluoranthene; 12 = benz(a)pyrene; 13 = dibenz(a,h)anthracene; 14 = benzo(g,h,i)perylene; 15 = indeno(1,2,3-cd)pyrene; and 16 = coronene.

For dilute solutions (< 0.05 AU) the observed fluorescence intensity is related to sample concentration by the expression $I_f = I_0 \phi_f (2.3 \epsilon l c)$, where I_f is the fluorescence emission signal, I_0 the source excitation intensity, ϕ_f the quantum yield (number of photons emitted/number of photons absorbed), ϵ the molar absorption coefficient, l the cell path length, and c the sample concentration. For typical conditions the detector response is linear over about two to three orders of magnitude. Sensitivity depends on the instrument (I_0 and the reduction of scattered and stray light), the sample (quantum efficiency), and the composition of the mobile phase (solvents, impurities, etc). The major sources of detector noise are specular scatter, Rayleigh and Raman scattering occurring in the flow cell material and mobile phase and luminescence impurities present in the flow cell materials and the mobile phase. Specular scatter results from the reflections and refractions of the excitation wavelength at the various optical interfaces of the detector.

Both the position of the emission wavelength envelope and the emission intensity can be affected by the mobile phase composition and even by the presence of contaminants, Table 5.3. Fluorescence detection is compatible with gradient elution unless one or more components of the mobile phase contain a high level of fluorescent impurities.

Flow cells for fluorescence detection with 3–10 μl volumes are similar in design to those used for absorption measurements. For microcolumn systems, the light output of conventional sources cannot be fully exploited because only a small fraction of the available excitation energy can be directed into the flow cell. If lasers are used as the source of excitation energy, a reduction in the cell volume can be achieved while maintaining an acceptable signal level [110,134]. Laser sources provide high intensity levels of collimated monochromatic light easily adapted to illuminating very small

Table 5.3
The effect of the mobile phase on fluorescence

Mobile phase	Fluorescence emission
pH	Both the emission wavelength and fluorescence intensity of ionizable compounds (i.e. those containing acidic or basic functional groups) are critically dependent upon pH and solvent hydrogen-bonding interactions.
Solvent	Intensity changes of an order of magnitude and large wavelength shifts occur for compounds which undergo strong solvent interactions. A shift in the fluorescence spectrum to longer wavelengths is usually observed with an increase in the solvent dielectric constant. If the solvent absorbs any of the excitation or emission energy, the sensitivity will be reduced.
Temperature	Many compounds show marked temperature dependence with increasing temperature causing a decrease in intensity of 1-2% per °C.
Concentration	At high concentrations the emission signal becomes non-linear due to self-absorption by the sample itself or complete absorption of the excitation energy before it can fill the full cell volume. High emission intensity may overload the photosensor resulting in an irregular and fluctuating signal.
Quenching	Impurities in the mobile phase, particularly oxygen, may quench the signal from low concentrations of fluorescent compounds (see solvent degassing).
Photodecomposition	High intensity sources may cause sample decomposition, which depends on the residence time of the sample in the detector cell.

volumes. When sample sizes are limited, for example, in some biological applications, laser-induced fluorescence may be the only technique suitable for detection. Most lasers provide only a limited number of discrete wavelengths, most of which are above 300 nm, and may not coincide with desirable excitation wavelengths for natural fluorescent compounds. Generally speaking, lasers provide a less stable light output than conventional sources and flicker noise limits analyte detectability. For conventional diameter columns and normal flow cells there is usually no advantage to using laser excitation sources over conventional arc sources.

Flow cells for packed capillary column systems are usually of two types. A flow cell can be fabricated from an unpacked portion of the fused-silica capillary by removing a section of the polyimide coating similar to those used for absorption measurements, except that the emission signal is generally measured at right angles to the direction of the excitation beam. In-column fluorescence detection is also possible using packed fused-silica capillary columns by removing a small section of the polyimide coating to create a viewing area before the column end frit [135,136]. The sample zones are now detected in the presence of the stationary phase eliminating band broadening from connecting tubes between the column and detector flow cell. In addition, the sample zones are focused, such that their concentration is increased by the factor $(1 + k)$, where k is the solute retention factor, compared to the concentration of the eluted

zone. There is also an environmental factor, which could result in an enhancement or diminution of the fluorescence emission for the adsorbed sample. On the other hand, it is likely that the excitation beam does not completely penetrate the column packing, so that only a fraction of the adsorbed analyte is excited and the presence of the packing causes additional scattering of the excitation beam manifested as an increase in background noise. In most cases, sample detectability does not seem to be very different for in-column detection compared with postcolumn detection, which is generally more convenient. Since light scattering and fluorescence impurities in the fused-silica tubing contribute to detector noise flow cells without walls have also been used [110]. Detection volumes in the low nanoliter range can be achieved with a sheath-flow cell, for example. In this approach the column eluent is ensheathed by a strong flow of solvent with a similar refractive index to the eluent, compressing it into a narrow stream a few μm in diameter.

5.7.1.3 Chemiluminescence Detectors

Chemiluminescence detection differs from fluorescence detection in that an exothermic chemical reaction is used to either transfer energy to a fluorescent compound or create a fluorescent product in an excited state, which later relaxes to the ground state releasing at least part of its excess energy as light [104,112,137,138]. Chemiluminescence detection is used to improve the sensitivity or selectivity of fluorescence detection. Since chemiluminescence does not require a light source detector noise from stray light and source instability are reduced providing for an increase in sample detectability of one to three orders of magnitude for the most favorable cases. But signal enhancement is not obtained for all reactions and the number of useful applications is restricted compared with natural fluorescence. For liquid chromatography it is convenient to consider detector options based on gas phase or liquid phase chemiluminescence reactions.

Gas phase chemiluminescence detectors are used for the selective detection of nitrogen-containing compounds [138-140]. Most nitrogen-containing compounds (except N_2) are converted to nitric oxide by reaction with oxygen at about 900-1100°C. Subsequent reaction of the nitric oxide with ozone at a reduced pressure produces electronically excited nitrogen dioxide, which relaxes to the ground state by emission of near-infrared radiation centered around 1200 nm. The column eluent is nebulized into a high temperature pyrolysis chamber supplied with a flow of oxygen. Complete combustion of the mobile phase and sample components in this chamber are essential. The pyrolysis gases consisting mainly of unconsumed oxygen, water, carbon dioxide and nitric oxide (from the oxidation of nitrogen-containing compounds) are routed through a membrane drying system to remove all water and then to the reaction chamber for quantification. The reaction chamber consists of a light-tight gas chamber where ozone and the pyrolysis gases mix producing a chemiluminescence emission when the pyrolysis gases contain nitric oxide. The emission is detected by a photomultiplier after passage through an optical filter to enhance the selectivity for the desired chemiluminescence signal. The detection limit for nitrogen-containing compounds is about 10^{-12} g

(N/s), selectivity 10^7 (g N/g C) with a linear response range of $10^4 - 10^5$. The equimolar response of the detector for nitrogen eliminates the need for authentic reference standards for all mixture components [141]. The detector is not well characterized at present and some questions concerning its ruggedness have been raised. There is less information available for the sulfur chemiluminescence detector (section 3.10.3.2) which has been used with packed capillary columns [142].

Liquid phase chemiluminescence detectors usually consist of a postcolumn reactor (section 5.8) connected to a fluorescence detector with its source disabled [104,137,138,143-145]. The column eluent is combined with one or several reagents that initiates the desired chemiluminescence reaction. The intensity of light emission depends on the rate of the chemical reaction, the efficiency of production of the excited state, and the efficiency of light emission from the excited state. The chemiluminescence intensity is sensitive to environmental factors such as temperature, pH, ionic strength, and solution composition. In addition, the detection system has to be designed to accommodate the time dependence of the chemiluminescence signal to ensure that adequate and representative emission occurs in the detector flow cell.

One of the most common reactions exploited for chemiluminescence is based on the hydrogen peroxide oxidation of an aryl oxalate ester, which produces a high-energy intermediate (1,2-dioxethane-3,4-dione) as the reactive species. Interaction of the 1,2-dioxetane-3,4-dione with a fluorescent compound results in its decomposition with energy transfer to the fluorescent compound. The excited fluorescent compound then emits light as it relaxes to the ground state. This reaction can be used to determine naturally fluorescent compounds and non-fluorescent compounds after formation of a fluorescent derivative, as well as some non-fluorescent compounds that are easily oxidized in the quench mode. Another common reaction is based on the quenching of the chemiluminescent reaction between luminol, an oxidant such as hydrogen peroxide, and a catalyst in an alkaline medium [146]. Under these conditions luminol is converted to 3-aminophthalate in an excited state that emits light at 425-435 nm. Because the chemiluminescence intensity is directly proportional to the concentration of luminol, peroxide and catalyst, species that can be converted into peroxides, species labeled with luminol or the catalyst, or species that interfere in the reaction mechanism can be quantified by monitoring the chemiluminescence emission.

5.7.2 Refractive Index

Refractive index detectors are bulk property detectors with a near universal response, albeit limited by poor sample detectability for some applications [101-106]. Differential detection is employed to minimize background noise with the result that the detector response is in some way related to the difference in refractive indices of the mobile phase and the mobile phase containing the sample. Consequently, an analyte will be detected only if its refractive index is different from that of the mobile phase. Peaks in both a positive and negative direction may be observed in the same chromatogram depending on whether the analyte has a higher or lower refractive index than that of

the mobile phase. Detector response factors are generally different for each component in a separation unless they have identical refractive index values. The sensitivity of the detector to variations in flow and temperature limit its use to isocratic separations.

The ultimate performance limit of the refractive index detector is caused by fluctuations in the refractive index of the mobile phase. The detector is sensitive to small changes in temperature and mobile phase composition, and the majority of problems associated with its practical operation can be traced to this cause. Changes in composition can occur from incompletely mixed mobile phases, leaching of prior samples or solvents from the column, or changes in the amount of dissolved gases in the solvent. The temperature and pressure dependence of the refractive index results in an offset in the detector baseline with changes in flow rate. Incomplete equilibrium of the incoming mobile phase temperature with the temperature of the detector flow cell and viscous heating in the inlet to the cell both contribute to this flow sensitivity. To be able to detect 10^{-6} to 10^{-8} g of solute, a noise equivalent concentration of 10^{-8} refractive index units is required. To maintain this noise level the temperature of the detection system must be thermostatted to within 0.001°C .

Commercially available refractive index detectors usually employ refraction, reflection or interference of a collimated light beam to sense differences in refractive index in sample and reference flow cells. The comparatively poor response and sensitivity to environmental factors restricts most applications to analytical separations using conventional diameter columns and preparative separations. Small flow cells suitable for use with packed capillary and small bore columns are easily fabricated and detectors based on reflection [147] and backscattering interferometry [148], for example, have been described for use with these columns. Poor concentration sensitivity and complicated detector operation, however, limit these detector systems. Other methods of refractive index difference measurements include surface plasmon resonance-based detectors [149] and spectroscopic refractometry [150]. The spectroscopic refractive index detector provides a novel method of correcting for thermal noise by measuring and comparing the refractive index response at two or more wavelengths simultaneously. This approach shows potential for improving detection limits in analytical refractive index detection.

The refraction-type refractive index detector (deflection-refractometer), Figure 5.16, measures the deflection of a collimated light beam when it crosses a dielectric interface separating two media of different refractive index at an angle of incidence other than zero (Snell's law). Light from a tungsten lamp passes through a beam mask, collimating lens, sample and reference cells; is reflected by a mirror; and passes back through the cells and lens, which focuses the light onto a position sensitive photosensor. A rotatable glass plate between the lens and photosensor is used to zero the detector signal when the sample and reference cells contain the same solvent. The sample and reference cells are triangular in shape and located in a single fused glass assembly. For analytical applications the cell volume is usually $10\ \mu\text{l}$. The incident light beam leaves the first cell and enters the second cell at 45° . If the refractive indices are the same in the two cells, the light beam leaving the first cell will be refracted the same amount but in the

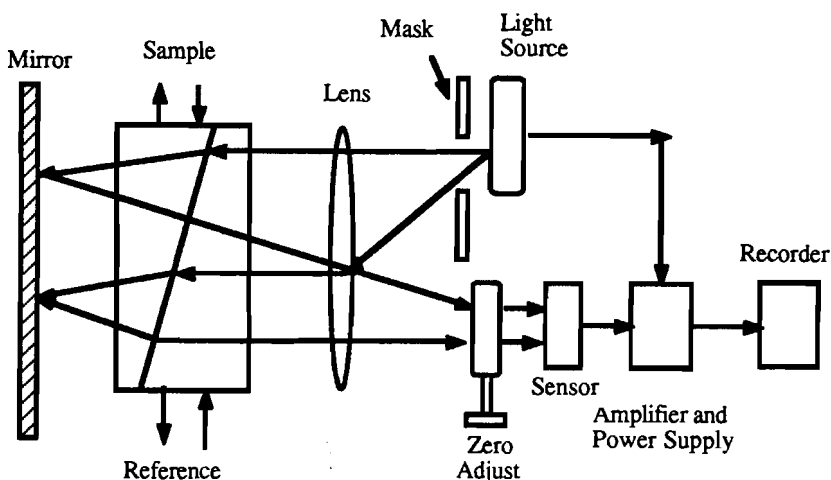


Figure 5.16. Schematic diagram of a refractive index detector employing the principle of refraction (deflection-refractometer).

opposite direction to that at which it enters the second cell. The net deflection of the light beam, therefore, is zero after passage through the two cells. If the refractive indices for the two cells are different, there will be a net deflection of the light beam after passage through the two cells, which is doubled after reflection from the mirror and passage back through the cells. This translates into a lateral displacement of the focused beam at the position of the photosensor. Deflection-refractometers have the advantage of a wide linear range, the entire refractive index range can be accommodated using a single cell, low volume cells can be fabricated, and the cells are relatively insensitive to air bubbles or the buildup of contaminants on the sample cell windows.

Reflection-type refractive index detectors, Figure 5.17, sense a change in the light intensity transmitted through a dielectric interface between the surface of a glass prism and the liquid to be monitored when the refractive index of the liquid changes. A beam mask and collimating lens are used to produce two parallel beams of light from a tungsten lamp that impinge on the prism-cell interface at slightly less than the critical angle for total internal reflection. The thin sample and reference cells are defined by the surface of the prism, the grained surface of the metal cell plate, which contains the inlet and outlet ports, and a thin PTFE gasket. A collecting lens focuses the light scattered from the grained metal surface on to a photosensor. The difference in intensity of the light reflected in the two beams is related to the refractive index difference between the sample and reference cell by Fresnel's law and has a fairly large linear range when the incident light strikes the cell near its critical angle. A disadvantage of this approach is that two different prisms are required to cover the full refractive index range of common chromatographic solvents. Advantages include high sensitivity, a small cell volume, and ease of cleaning.

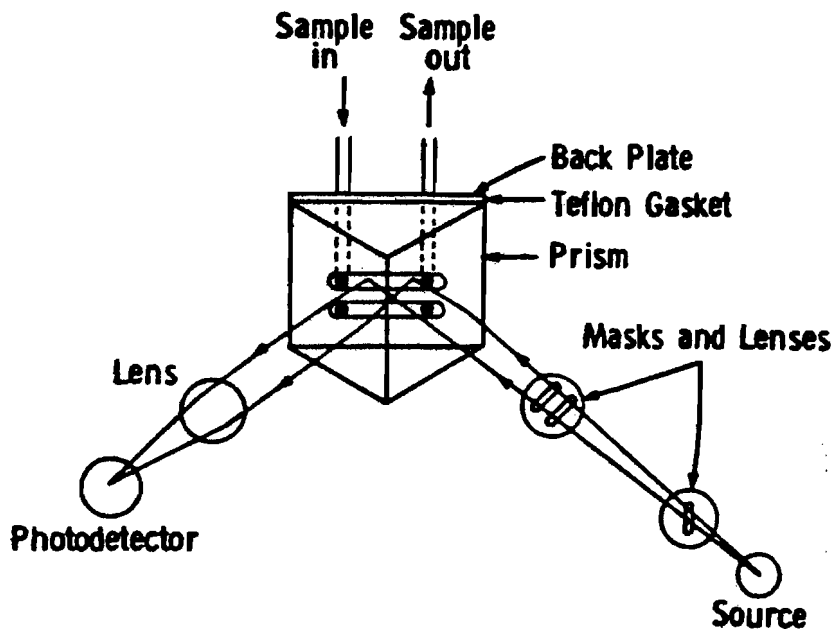


Figure 5.17. Schematic diagram of a refractive index detector employing the principle of reflection (Fresnel-refractometer).

The interferometric refractive index detector, Figure 5.18, measures the difference in the effective path length (speed of light) of a light beam passing through sample and reference cells by means of the interference of the two beams when recombined at a photosensor. By definition the speed of light is directly proportional to the refractive index of a medium. As the relative speed of light in the two cells changes, destructive interference occurs and the light intensity at the photosensor decreases. To maximize sensitivity, the light beam is first linearly polarized, and the horizontal and vertical polarized components used for the sample and reference light beams, respectively. It is likely that the sensitivity of this detector is similar to that of the reflection and refraction based detector when used with conventional diameter columns.

5.7.3 Evaporative Light Scattering

The evaporative light-scattering detector (ELSD) is a near universal detector suitable for the determination of (mainly) neutral compounds that are less volatile than the mobile phase used for the separation [151,152]. Primary uses include the detection of compounds with a weak response to the UV detector, especially carbohydrates, lipids, surfactants, polymers and petroleum products. Its greater sensitivity and ease of use in gradient elution separations makes it preferable to the refractive index detector for these applications. The ELSD is compatible with most volatile solvents used for normal and

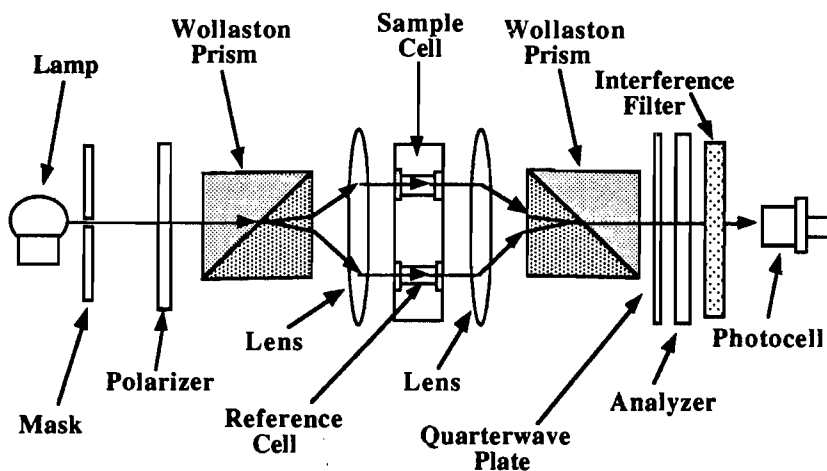


Figure 5.18. Schematic diagram of an interferometric refractive index detector.

reversed-phase separations. Typical limits of detection are about 1 ng corresponding to a sample concentration of about 1 $\mu\text{g} / \text{ml}$ for conventional diameter columns and about 10 $\mu\text{g} / \text{ml}$ for packed capillary columns. Its main drawback for quantitative analysis is the non-linear relationship between the detector response and the sample mass [153-157]. At low sample concentrations the detector response is described by the relationship $A = am^b$ where A is the peak area, m the analyte mass and a and b are coefficients that depend on the detector operating conditions and analyte characteristics. The b coefficient generally takes values between 0.9 and 1.8. Even though the response is non-linear it will usually have some range in which the response appears linear. The linearity of the detector response can be extended by logarithmic transform of the response function. Since the coefficients a and b are compound and system dependent calibration is required for quantification.

The three main processes that occur successively with the ELSD are: conversion of the column eluent to an aerosol (nebulization); evaporation of volatile components from the aerosol droplets in a heated drift tube to form particles (desolvation); and determination of the particle density by light scattering in a gas flow cell. Pneumatic nebulizers are commonly used for aerosol generation. The column eluent is mixed with a gas stream in a concentric tubular nebulizer forming a high-velocity jet of tiny liquid droplets. These droplets either proceed directly into the drift tube or a separate spray chamber connected to the drift tube where the larger droplets are removed by condensation. The use of a spray chamber generates a more homogeneous aerosol and allows the drift tube to be operated at a lower temperature for desolvation. The low flow rates typical of packed capillary columns require replacement of the standard nebulizer by a miniaturized version [158-162]. For example, by a fused-silica restrictor (e.g. a length of 20 μm internal diameter capillary tube) housed in a wider bore stainless

steel capillary with nitrogen gas flowing through the space between the two capillaries. Insertion of the miniaturized nebulizer into the end of the drift tube and its contact with the heated wall of the drift tube, the purpose of which is to preheat the nebulizer gas, allows faster system equilibration at startup and production of a more stable and homogeneous aerosol.

The aerosol is next desolvated by evaporation of volatile mobile phase components by contact with warm gas as it traverses the short thermostatted drift tube. The temperature required to dry the droplets depends on the evaporation properties of the mobile phase and the droplet size. Typical temperatures are between 30 to 80°C. It is important to use as low a temperature as possible for this process to maintain particle size uniformity and to encourage formation of larger crystalline particles, which maximize the intensity of scattered light. In addition, too high a temperature may result in the complete or partial loss of the more volatile analytes from the chromatogram. At the end of the drift tube, a light beam is scattered by the particles present in the gas flow. The intensity of the scattered light is measured by a photosensor located at an angle to the incident beam direction. The intensity of the scattered light depends on the size distribution of the particles formed in the drift tube, which in turn depends on the size of the droplets formed during the nebulization process. The addition of a chamber for condensation nucleation between the drift tube and light scattering cell, which has the effect of increasing the average particle size, has been shown to provide a significant increase in detector response [151,163,164]. Mobile phases that contain non-volatile components cause an elevated background and decrease sample detectability as well as a rapid degradation of detector performance, due to their deposition in the optical cell. Mobile phase additives and buffers are limited to such species as volatile acids (formic, acetic, trifluoroacetic and nitric acids), volatile bases (e.g. triethylamine, ammonia, pyridine) and volatile salts (ammonium bicarbonate, ammonium acetate, ammonium formate).

5.7.4 Electrochemical

Electrochemical detectors based on the principles of capacitance (dielectric constant detector), resistance (conductivity detector), voltage (potentiometric detector) and current (amperometric, coulometric and polarographic detectors) have all been used in liquid chromatography [165-171]. Conductance is a universal property of ions in solution. It is an obvious choice for the continuous and selective monitoring of ionic species in an aqueous mobile phase. The prominence of the conductivity detector is tied to the acceptance of ion chromatography for the analysis of common ions with poor UV absorption properties. Amperometric detection is based on the oxidation or reduction of an analyte at a working electrode held at a potential sufficient to initiate the electrode reaction. The electric current resulting from the reaction is proportional to the concentration of the analyte in the column eluent. Some selectivity in the detection process is possible based on the structure-related voltages required for the electrolysis of individual functional groups and ions. Electrolysis is usually

incomplete, about 1-10%, for typical detection conditions. By increasing the electrode surface area the electrolysis reaction may reach (near) completion, corresponding to the coulometric limit. The response of the coulometric detector is absolute, eliminating the need for calibration. On the other hand, although the conversion of electroactive species is higher, the background noise is also greater, and sample detectability is generally similar for coulometric and amperometric detectors. The potential applied to the working electrode is usually constant throughout the separation or applied as a pulse waveform. Triple-pulse and other waveforms are often used when products of the electrochemical reaction contaminate the electrode surface. The successive and repetitive application of a measuring potential, a cleaning potential and a conditioning potential at a noble metal electrode is the basis of pulsed amperometric detection. This is the most important of the pulsed electrochemical detection techniques and is used to extend the application range of amperometric detection to polar aliphatic compounds (e.g. carbohydrates, surfactants, etc). Polarographic detectors, employing a dropping mercury electrode, are used less frequently than solid-electrode amperometric detectors in liquid chromatography. Their sensitivity is similar to solid-electrode amperometric detectors but their application range is restricted to reducing species (the low oxidation potential of mercury precludes its use for oxidizable species). Potentiometric detectors (ion selective electrodes) are too specific for general applications in liquid chromatography [167,172].

5.7.4.1 Conductivity Detectors

Conductivity detectors have a simple and robust design and are easily miniaturized [102,106,168,172-175]. A typical detector consists of two electrodes housed in a chamber fabricated from a non-conducting material with a volume of 1 – 10 μ l. The electrodes are of low surface area and constructed of an inert conducting material, such as stainless steel or platinum. The cell resistance is usually measured in a Wheatstone bridge circuit. During the measurement of the cell resistance, undesirable processes, such as electrolysis or the formation of an electric double layer at the electrodes, may occur. By varying the frequency of the applied potential these undesirable effects are suppressed or completely eliminated. Some detectors apply a sinusoidal wave potential across the cell electrodes at 1-10 KHz and synchronous detection of the component of the cell current which is in phase with the applied potential frequency. Alternatively, a bipolar pulse technique consisting of two successive short voltage pulses of opposite polarity but equal amplitude and duration can be used. At exactly the end of the second pulse the cell current is measured and the cell resistance is determined from Ohm's law. The detector cell is thermostatted to avoid sudden temperature changes in addition to a temperature-compensating circuit to adjust the cell resistance to the temperature of the column eluent. All detectors have some means of correcting for the background conductivity of the mobile phase, which limits the absolute sample detectability. In the absence of significant background conductivity detection limits of 10^{-8} – 10^{-9} g / ml are possible.

If a conductivity detector is used to monitor the effluent from an ion-exchange column to separate anions, for example, then the change in observed conductivity, ΔG (expressed in microsiemens), as the analyte peak passes through the conductivity cell is given by

$$\Delta G = (\lambda_{A-} - \lambda_{E-})C_A\alpha_A / 10^{-3}K \quad (5.3)$$

where C_A is the concentration of analyte anions, α_A the fraction of analyte present in the ionic form, λ_{E-} and λ_{A-} the limiting equivalent ion conductance of the competing eluent anion and analyte, respectively, and K the cell constant that takes into account the physical dimensions of the cell [168,176,177]. Useful sample detectability requires a large difference in the limiting equivalent ionic conductance of the analyte and eluent ions. This difference can be positive or negative, depending on whether the eluent ion is strongly or weakly conducting. When the mobile phase ions have a higher equivalent conductance than the analyte ions the method of detection is referred to as indirect conductivity detection.

The most common use of conductivity detection is in single column and suppressed ion chromatography (section 4.3.7.2). Single column ion chromatography is achieved by using dilute solutions of electrolyte with a high affinity for the stationary phase and a suitable difference in equivalent conductivity to the analyte ions for adequate sample detectability as a mobile phase. Suppressed conductivity detection is based on the chemical amplification of the conductance of the analyte ions with suppression of the conductance of eluent ions prior to conductivity detection. For this purpose weak acid (or base) salts are used as eluent competing ions for the elution of anions (or cations) followed by exchange of the eluent ions using an ion-exchange resin or membrane for hydronium ions (hydroxide ions for cations). This process results in a reduction of the conductance of the eluent by replacing the high-conductance eluent ions by a partially ionized weak acid (or weak base). Simultaneously, the conductance of analyte ions, which are strong acids (or strong bases) is increased by pairing them with highly conducting hydronium ions (or hydroxide ions for cation determinations). For example, suppressed conductivity detection of chloride and nitrate uses eluents containing sodium hydroxide or carbonate-bicarbonate buffers that can be converted into species of low conductance (H_2O and H_2CO_3 in this case) after exchanging the cations of the eluent (sodium) for hydronium ions by a suitable cation-exchange device. In the same process the analyte ions are converted to species of higher conductance by replacing sodium ions by hydronium ions (HCl and HNO_3). Originally, a packed column containing a high capacity ion-exchange resin in the hydrogen or hydroxide form located in the eluent flow path between the separation column and the conductivity detector was used for suppressed conductivity detection. This arrangement, however, was not ideal and it is more common to use ion-exchange membrane suppressors today [168,173, 178-180].

The main disadvantages of packed-column suppressors are: that they require periodic regeneration due to limited capacity; result in significant extracolumn band broadening,

since reasonable suppressor capacity requires relatively large suppressor columns; weak acids and bases exhibit variable retention resulting from several mechanisms, which change with the degree of suppressor exhaustion; and the “water dip” resulting from the elution of the sample solvent often hampers trace analysis of some analytes that are eluted early in the chromatogram. Some of these problems were solved by the introduction of hollow-fiber suppressors based on ion-exchange polymeric membranes [168,181]. The main advantage of the hollow-fiber ion-exchange suppressor was that it allowed continuous operation of the ion chromatograph by exchanging ions across the membrane wall into a continuously regenerated solution of electrolyte bathing the fibers. Relatively large dead volumes, limited suppression capacity, and the fragile nature of the fibers made them less than ideal. Eluent suppression capacity was largely limited by slow mass transfer of ions to the membrane wall, and although second generation hollow-fiber suppressors filled with beads or fibers and shaped into various helical forms to promote turbulence improved ion transport to the membrane wall, they lack equivalent performance to the micromembrane suppressors now in common use.

The micromembrane suppressor, Figure 5.19, combines the high ion-exchange capacity of packed-bed suppressors with the constant regeneration feature of hollow-fiber suppressors in a low-dead volume ($< 50 \mu\text{l}$) configuration [168,173,178-182]. The eluent from the separator column flows between two thin ion-exchange membranes that are separated by an intermediate screen made from a polymer with ion-exchange sites. A solution of electrolyte is pumped countercurrently and externally to the membrane in small volume channels that are partially filled with polymer screens. The purpose of the screens is to enhance the transport of ions to the ion-exchange membrane by generating turbulent flow and by providing a site-to-site path for transport of ions to the membranes (screens in the eluent chamber contain ion-exchange sites). The mechanism of suppressor operation for anion and cation detection is illustrated in Figure 5.20. The micromembrane suppressor has a higher suppression capacity than other suppressor designs. It is compatible with the use of higher buffer concentrations, higher eluent flow rates and facilitates applications requiring composition gradients for convenient separation. For weak acids and bases the micromembrane suppressor can be used to exchange metal cations to enhance conductivity by formation of conducting salts [183,184]. In addition, it can be used as a device for pH control in a reaction detector configuration [182].

Significant advances in suppressor technology with respect to convenience of operation have been made in the last few years using electrolytic suppressors and solid-phase chemical regeneration [178,179,185-188]. A disadvantage of membrane suppressors is that they require an additional pump and a supply of regenerant solution for continuous operation. Electrolytic suppressors replace the regenerant solution with water that is electrolysed in the regenerant compartments of the micromembrane suppressor or separate regeneration cells to produce the hydronium and hydroxide ions necessary for the suppression reaction. In self-regenerating suppressors the deionized eluent waste from the suppressor is recycled from the detector to the regenerant chamber. The amount of water available for sweeping out products from the suppressor

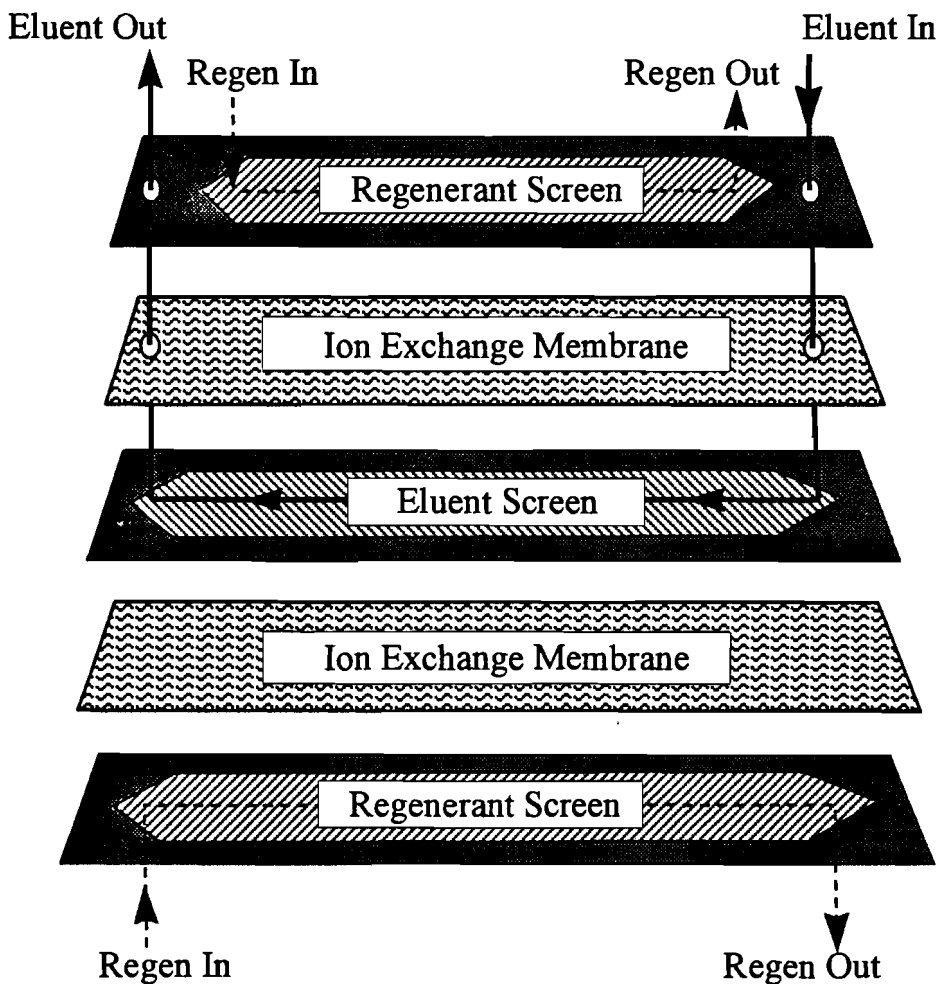


Figure 5.19. Exploded view of a micromembrane suppressor (regen = regenerant solution). (From ref. [182]; ©Elsevier).

operation is now limited to the eluent flow rate, resulting in a higher, although acceptable background noise for most applications, when compared to systems using an external source of water. Self-regenerating suppressors can be used with solid-phase regenerant cartridges in the recycle line to strip suppressor waste products from the regenerant supply. The cartridges provide a visual indication of exhaustion and can be used without electrolytic suppression. Organic solvents are not well tolerated by electrolytic suppressors and chemical regenerant methods are preferred for applications employing partially aqueous mobile phases.

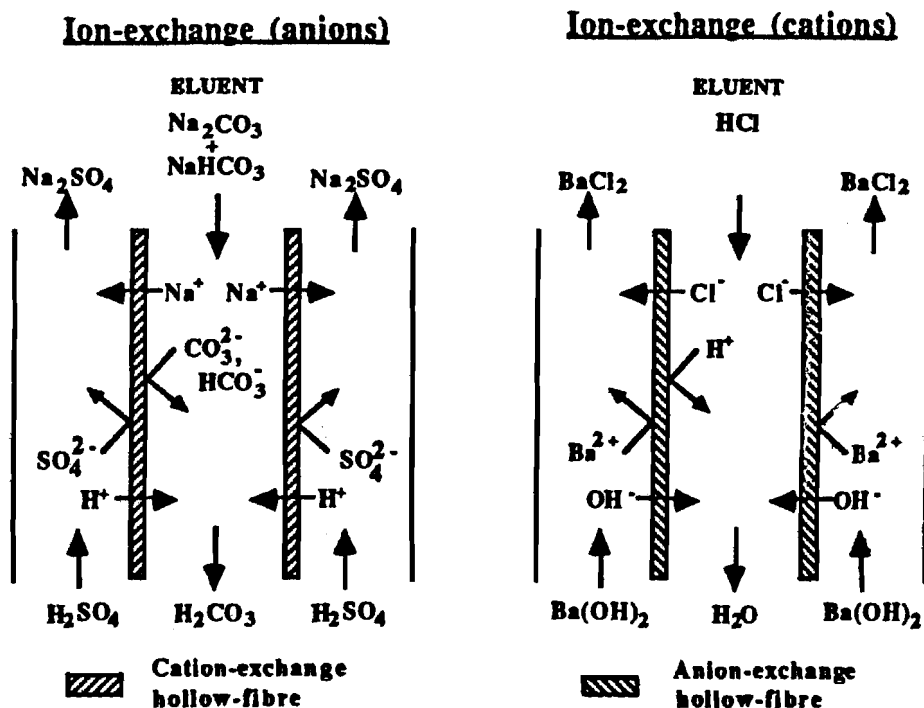


Figure 5.20. Schematic representation of the operation of a membrane suppressor for suppressed conductivity detection of anions and cations. (From ref. [168]; ©Elsevier).

5.7.4.2 Constant Potential Amperometric Detectors

The constant potential amperometric detector determines the current generated by the oxidation or reduction of electroactive species at a constant potential in an electrochemical cell. Reactions occur at an electrode surface and proceed by electron transfer to or from the electrode surface. The majority of electroactive compounds exhibit some degree of aromaticity or conjugation with most practical applications involving oxidation reactions. Electronic resonance in aromatic compounds functions to stabilize free radical intermediate products of anodic oxidations, and as a consequence, the activation barrier for electrochemical reaction is lowered significantly. Typical applications are the detection of phenols (e.g. antioxidants, opiates, catechols, estrogens, quinones) aromatic amines (e.g. aminophenols, neuroactive alkaloids [quinine, cocaine, morphine], neurotransmitters [epinephrine, acetylcholine]), thiols and disulfides, amino acids and peptides, nitroaromatics and pharmaceutical compounds [170,171]. Detection limits are usually in the nanomolar to micromolar range or 0.25 to 25 ng / ml.

A number of electrochemical cell designs have been described but the most popular configurations are the three-electrode thin-layer cell and the wall-jet cell, Figure 5.21[20,102,166-171,189]. The column eluent is introduced either parallel to

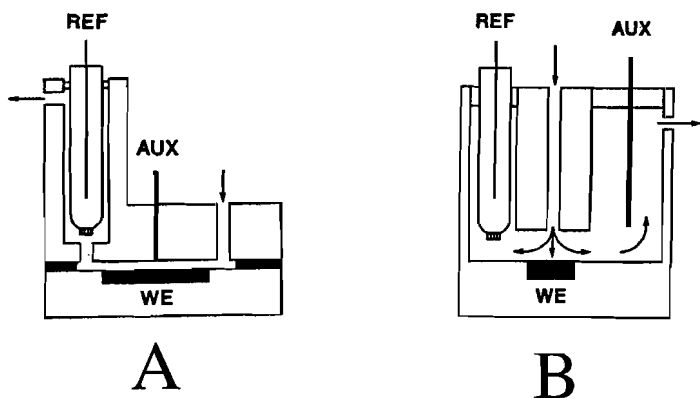


Figure 5.21. Schematic representation of a thin-layer electrochemical cell (A) and a wall-jet electrochemical cell (B). AUX = auxiliary electrode, REF = reference electrode and WE = working electrode.

the working electrode embedded in the channel wall using the thin-layer cell, or perpendicular to the working electrode surface followed by radial dispersion in the wall-jet design. The cell body is usually made out of an insulating material like PTFE and machined to accommodate the eluent channel and various electrodes. The working electrode, usually in the form of a disk, is embedded in the wall of a channel defined by a gasket sandwiched between two blocks. A typical cell volume is about 1-10 μl . The reference and auxiliary electrodes are placed at the downstream side of the working electrode, so that leakage from the reference electrode or formation of electrolysis products at the auxiliary electrode does not interfere with the working electrode. Inclusion of a reference electrode allows the potential of the working electrode to be set at a predetermined value suitable for electrolysis of the analytes. The chosen potential is selected to operate the cell in the steady state limiting current region, and can be deduced from hydrodynamic voltammograms. The auxiliary electrode serves as a reference for current measurement at the working electrode. As part of a current feedback loop the auxiliary electrode can inject or remove charge from the cell to maintain a constant potential at the working electrode. The types of materials used for the reference electrode (e.g. silver-silver chloride) and auxiliary electrode (e.g. platinum or stainless steel) are not critical, but the choice of working electrode material is very important as it affects detector performance. A wide variety of materials have been used for the working electrode, but the most popular is glassy carbon. Glassy carbon has a surface that is easily polished, inert to all solvents used in liquid chromatography and with a working range of -1.3 to +1.5 V, it is suitable for a wide variety of applications. Metal electrodes are generally too easily oxidized producing high residual currents for general use in detector flow cells. The large negative potential range of the silver/mercury amalgam electrode makes it suitable for reduction reactions. Porous electrode cells because of the high surface area of the working electrode are popular for coulometric detection. The working electrode is usually made from a composite of

carbon particles sufficiently permeable to allow the column eluent to flow through the electrode. These cells are difficult to clean and have a high background noise limiting their use for amperometry.

Amperometric detectors are easily miniaturized with preservation of performance, since their operation is based on reactions at the electrode surface. Using a single carbon fiber or microelectrode as a working electrode allows detector cells of very small volume and in-column detectors to be constructed for use in open tubular and packed capillary column liquid chromatography [189-192]. These microcolumn separation techniques combined with amperometric detection are exploited for the quantitative analysis of volume-limited samples such as the contents of single cells [193,194].

The chromatogram is recorded by measuring the detector cell current at a fixed potential as the sample is eluted from the column. The background will remain constant as long as the mobile phase velocity and composition do not change and is subtracted from the analytical signal. The resulting detector current is directly proportional to the concentration of electroactive species in accordance with Faraday's law. Detector operation is critically dependent on flow rate constancy, solution pH, ionic strength, temperature, cell geometry, the condition of the electrode surface and the presence of electroactive impurities (dissolved oxygen, halides, trace metals, etc.) [20,169-171]. The background detector noise and, thus the ultimate sensitivity of the detector, are controlled by dissolved oxygen, ionic impurities and the contamination of the electrode surface, coupled with transient changes in the eluent flow rate. Gradient elution is not normally possible. Amperometric detection requires the use of mobile phases containing salts or mixtures of water with water-miscible organic solvents, conditions that are compatible with reversed-phase and ion-exchange chromatography, but are more difficult to achieve with other separation modes. These problems can be circumvented, to some extent, if the mobile phase is water miscible by adding a makeup flow of support electrolyte at the column exit.

Amperometric detectors containing (usually) two or more working electrodes have been developed to improve the overall selectivity or sensitivity of the detection process [20,102,189]. The two working electrodes can be arranged in parallel or series. The peak height ratio at two different potentials for electrodes in parallel provides information on the identity of a peak or an estimate of its purity. Using the series configuration compounds eluted from the column can be oxidized (or reduced) on the first electrode and subsequently reduced (or oxidized) on the second. Since the electroactive compounds that irreversibly react on the first electrode are eliminated, determination of a reversible electroactive compound is possible with high selectivity. This technique can be useful for the removal of oxygen interference in which the upstream electrode reduces oxygen and the analyte, and the downstream electrode oxidatively detects only the reduction product of the analyte. Lithographic techniques allow the construction of microelectrode arrays containing a large number of adjacent micron-size electrodes on a single substrate [195,196]. One application of these arrays is enhancement of sample detectability by redox cycling across adjacent oxidizing and reducing electrodes.

5.7.4.3 Pulsed Amperometric Detectors

Pulsed amperometric detection is used for the direct detection of a variety of polar aliphatic compounds, many of which, like carbohydrates, peptides and sulfur-containing compounds are of biological interest [171,197-200]. Most aliphatic compounds are not amenable to constant potential amperometric detection. Free-radical products from the oxidation of aromatic molecules can be stabilized by π -resonance; hence the activation barrier for reaction is decreased. This mechanism is unavailable for stabilizing aliphatic free radicals. The activation barrier for oxidation of aliphatic compounds can be decreased at noble-metal electrodes with partially unsaturated d-orbitals (e.g. gold, platinum) that can adsorb and thereby stabilize free radical oxidation products and intermediates. Carbon electrodes are not electrocatalytic and are unsuitable for pulsed amperometric detection.

Pulsed amperometric detection exploits the high electrocatalytic activity of noble-metal electrodes by combining amperometric detection with pulsed potential cleaning of the electrode surface. Surface-adsorbed products produced during the short detection potential step are efficiently desorbed from the noble-metal electrodes by application of a large positive-potential pulse with concurrent formation of a surface oxide layer. The oxide-covered electrode is inert and must be reduced by a negative-potential pulse to desorb the oxide layer and restore the activity of the noble-metal surface. By utilizing a simple three-step potential waveform with a frequency of 0.5-2 Hz, Figure 5.22, a reproducible response with favorable detection limits is obtained during chromatographic separations. By monitoring the transient current at the same delay time in the detection cycle a Faraidic signal with a reasonable linear dependence on the analyte concentration is obtained. Selection of potential values for the pulse waveform is identified from cyclic voltammetry. Mobile phase characteristics (e.g. pH, ionic strength and type and concentration of organic modifier) affect the detector response characteristics. Organic solvents can be tolerated only if they are not electroactive for the conditions of the pulsed-potential waveform. Electroinactive additives adsorbed by the electrode can affect baseline stability if they interfere in the formation of the oxide layer. The pH of the eluent is important for controlling the amplitude of the detector response. Increasing pH shifts the potential for oxide formation to more negative potentials from the optimized value for detection. Separations employing a pH gradient are possible by replacing the reference electrode by a pH electrode to continuously adjust the detection potential throughout the separation. All alcohol-based compounds (e.g. carbohydrates) are determined by direct oxidation at oxide-free surfaces with a potential less than about 200 mV at gold electrodes in alkaline solutions or platinum electrodes in acidic solutions. Aliphatic amines and amino acids are detected at gold and platinum electrodes in both alkaline and acidic solutions at potentials greater than 150 mV at which concurrent formation of surface oxide promotes electrode reactions. Numerous sulfur-containing compounds can be detected under similar conditions. Sulfur-containing compounds and inorganic ions can also be detected under conditions selected to interfere in the formation of the surface-oxide layer in an indirect detection mode.

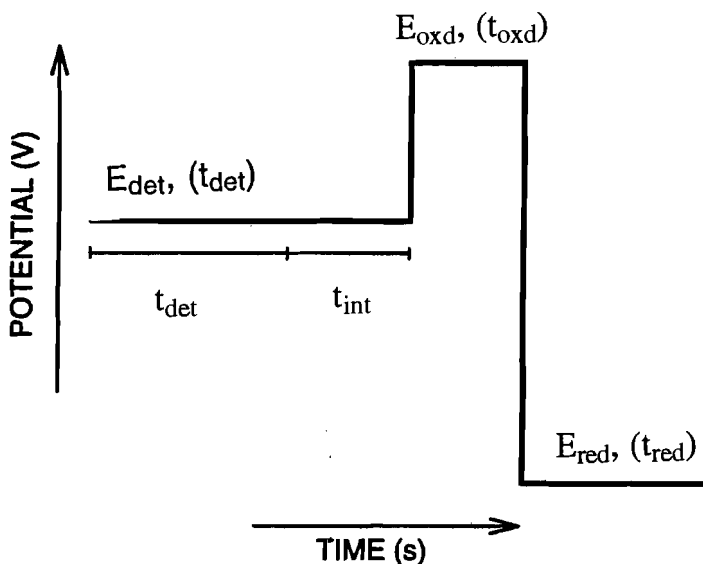


Figure 5.22. Three-step potential-time waveform for pulsed amperometric detection at a noble-metal electrode. E_{det} is the appropriate potential for the desired surface-catalyzed reaction applied for a time t_{det} composed of a delay time, t_{del} , and a short period at the end of the detection period, t_{int} , during which the current is sampled. Following the detection process, the electrode is cleaned by oxidative desorption concurrent with surface oxide formation using a step change in the applied potential to E_{oxd} for time t_{oxd} . The oxide-coated electrode is reactivated by a negative step potential E_{red} for time t_{red} sufficient to remove the oxide layer prior to the next cycle of the waveform.

Limits of detection for pulsed amperometric detection vary widely depending on the analyte and operating conditions. For normal-sized injections, limits of detection for compounds with a favorable detector response are usually in the pM to nM range ($\mu\text{g} / \text{ml}$ to ng / ml sample concentrations) with a linear response range of about 1000 fold.

5.7.5 Miscellaneous Detectors

From the perspective of system requirements detection has long been considered the weak link in liquid chromatography, especially when compared with the ability of gas chromatographic detectors to function across the range of normal application needs for gas chromatography. This has maintained interest in the development of new detection principles for liquid chromatography, some of which have resulted in commercial devices for specific applications. In addition, mass spectrometry is emerging as a useful detection technique for liquid chromatography as well as a source of structural information for identification (section 9.2). Viscometry and low-angle laser light scattering detectors for absolute molecular weight estimations in size-exclusion chromatography are discussed in section (4.3.9.3).

Given the success of ionization detectors in the field of gas chromatography it is no surprise that their adaptation to liquid chromatography has attracted considerable interest, although arguably without great success [201]. The two common approaches to interface liquid chromatography to gas phase ionization detection are based on transport systems and direct eluent introduction as a droplet stream (eluent-jet interface) or aerosol (nebulizer interface). In general ionization detectors are incompatible with typical eluent flow rates and solvents commonly used in liquid chromatography. Transport detectors attempt to solve this problem by eliminating the mobile phase by assisted evaporation prior to detection. Direct introduction methods rely on the tolerance of some ionization detectors to vapors generated from the low flow rates ($\mu\text{l} / \text{min}$) characteristic of packed capillary columns.

A transport detector consists of a carrier (e.g. wire, disk or chain) that continuously passes through a series of separate cleaning, coating, evaporation and pyrolysis chambers [202,203]. In the cleaning chamber all volatile material is removed from the carrier by heating it to about 750°C before it enters the coating chamber. In the coating chamber a jet applies a fraction of the eluent as a thin film to the carrier. In the evaporation chamber a controlled temperature and counter flow of gas strips the volatile mobile phase from the less volatile sample. In the pyrolysis chamber a controlled temperature is used to volatilize the sample into the detector, or into a chemical reactor for conversion to an easily detectable product. Modern versions of the transport detector use an oxidized titanium ribbon 1.5 mm wide and 0.1 mm thick as a carrier, which has a higher coating capacity than earlier versions employing stainless steel wires or metal chains. At a velocity of 3.5 mm/s the coating capacity of the ribbon is about 0.18 ml / min. Operation is restricted to separations obtained with volatile mobile phases. It can be configured to operate with a number of ionization detectors based on sample volatilization or decomposition occurring in the pyrolysis chamber. The rather complex operating mechanism, noisy signal and moderate sensitivity are considered the main drawback of transport detectors.

Packed capillary columns can be interfaced directly to flame-based ionization and photometric detectors or flameless thermionic ionization and electron-capture detectors using an eluent-jet or nebulization liquid introduction system [201,204,205]. The eluent-jet interface generates a jet of micron-sized droplets by application of a sharp temperature gradient at the tip of the introduction capillary. The capillary is cooled by a coaxial flow of gas while the eluent is heated inductively by an external radio frequency generator. The nebulization interface mixes the eluent stream with gas and sprays the liquid directly into the detector or a separate chamber that allows the larger droplets to settle out creating a more uniform aerosol. Both organic and aqueous eluents can be used with element-selective detectors. Sample detectability and linear response ranges are reasonable if not as good as when the same detectors are used in gas chromatography (e.g. TID $< 10 \text{ ng} / \text{s}$ N, FPD $20 \text{ pg} / \text{s}$ S, ECD $5\text{--}10 \text{ pg}$ all with a linear response range of about 10^3). The limited injection volumes of packed capillary columns results in less impressive concentration detection limits. The flame ionization detector can not be used with mobile phases containing a significant amount

of organic solvent but is a useful detector for separations employing hot pressurized water as a mobile phase (section 7.7.2).

Studies on chemical speciation in the environment and biology require a separation step combined with element selective detection. Many inorganic and organometallic compounds are easily separated by reversed-phase, ion-pair, ion-exchange and size-exclusion chromatography but the selectivity of common detectors is often inadequate for typical practical applications. In these studies, element-selective detection has generally been achieved by coupling liquid chromatography with flame, furnace and plasma atomic emission and atomic absorption spectrometers [206-210]. Contemporary interest is centered on the use of inductively coupled plasma-mass spectrometry (ICP-MS) as a universal element and isotope detector [210-213]. The ICP is a well-characterized, high temperature source suitable for the atomization and ionization of elemental species. The plasma is an electrodeless atmospheric pressure discharge created in a stream of (usually) argon sustained by energy from a radio frequency generator. Advantages of ICP-MS as a chromatographic detector include a wide linear range, low limits of detection (ng / l to $\mu\text{g / l}$), multielement and isotope analysis capability, and simple ion spectra. The mass spectrometer may be set to monitor the isotopic signal of an element, or several elements, with respect to time resulting in a chromatogram that contains peaks only for the elements of interest. The coupling of liquid chromatography to ICP-MS is straightforward and only requires a short length of inert polymeric or stainless steel tubing to connect the column to the nebulizer of the ICP. The choice of nebulizer is dictated by the mobile phase flow rate. Liquid flow rates of about 1 ml / min are compatible with conventional cross flow and concentric pneumatic nebulizers with single pass, double pass and cyclone-type spray chambers [214,215]. Conventional nebulizers provide low sample utilization with only about 1-3 % of the sample entering the nebulizer reaching the plasma. Small though significant improvements in sample utilization have been achieved using cooled spray chambers, hydraulic high-pressure nebulization, ultrasonic nebulizers and thermospray introduction systems. For liquid flow rates of $10\text{-}120 \mu\text{l / min}$ direct injection nebulization without a spray chamber is used. Both aqueous and organic solvents and their mixtures can be handled fairly easily by an appropriate combination of liquid flow rate, spray chamber temperature and radio frequency power supplied to the plasma. High proportions of organic solvents can lead to deposition of elemental carbon at the ion entrance to the mass spectrometer resulting in unstable and irreproducible ICP-MS operation. The addition of oxygen to the nebulizer gas and an increase in radio frequency power to the plasma can be used to minimize this problem. The type of organic solvent used for the separation is usually selected based on its ability to provide an adequate separation and by its effect on the ICP [216]. Mobile phases with a high salt content ($> 2\% \text{ w/v}$) should be avoided. In general, polyatomic spectral interferences are the most serious problem in ICP-MS (e.g. $^{40}\text{Ar}^{35}\text{Cl}^+$ on the determination of $^{75}\text{As}^+$, Ar_2H^+ on the determination of $^{81}\text{Br}^+$, etc). Polyatomic spectral interferences mainly occur in the mass region below 100 where many non-metallic elements of interest for the selective detection of organic compounds

occur. A collision reaction cell containing a mixture of helium and hydrogen can be used to eliminate interference from argon-containing ions for reliable detection [217].

The use of flow-through radioactivity detectors to study the metabolism or degradation of mostly ^{14}C , ^3H , ^{35}S and ^{32}P labeled compounds *in vivo* or environmental systems is widely used in some industries and university laboratories. Complexes containing the γ -emitters ^{125}I and $^{99\text{m}}\text{Tc}$ are used in nuclear medicine. All these radionuclides can be determined by the secondary photons produced by the interaction of energetic particles with a suitable photon emitter (scintillator). The column eluent is usually either mixed with a suitable scintillation cocktail prior to the detector cell or is passed through a transparent tube packed with a suitable solid-phase scintillator housed in the detector cell [106,218-220]. Two photomultiplier tubes at opposite sides of the detector cell are used for photon counting. The output of the photomultiplier tubes is processed using coincidence electronics. In this way noise spikes and other electronic interferences sensed at both photomultipliers simultaneously are rejected. The output from the coincidence electronics passes to a multichannel pulse height analyzer and then to a computer for data collection and display. Operational characteristics are usually indicated as disintegrations per minute (dpm) with typical detection limits of 300-500 dpm for ^{14}C and 1000-1500 dpm for ^3H . Limits of detection are related to the counting time (the residence time of the sample in the detector flow cell). For increased sample detectability it may be preferable to collect the column eluent in a fraction collector and count fractions off-line using a conventional scintillation counter [220,221]. The handling of radioactive materials requires special facilities and dedicated equipment, which may become contaminated by samples, and is rarely attempted in general analytical laboratories.

Chiral-selective detection based on polarimetry or circular dichroism provides an alternative to enantiomeric-selective separation systems for identifying and quantifying individual enantiomers in racemic mixtures [222-227]. Polarimetry and circular dichroism detectors respond directly to the intrinsic optical activity of a compound with a response to each enantiomer that is equal in magnitude, but opposite in sign. These differences are based upon the differential response of a chiral compound to polarized light. In polarimetry the difference in refractive index for right versus left circularly polarized light is measured at a single wavelength generated by a laser to improve sensitivity and facilitate the use of small detector volumes. When passed through an optically active medium (e.g. sample in a flow cell) one circularly polarized component will exhibit retardation relative to the other. The change in the phase between the two recombined components manifests itself as a rotation in the plane of polarization specified by an angle. In circular dichroism the differential absorbance of a chiral system for right and left circularly polarized light is recorded over a range of wavelengths (220-500 nm) isolated by a scanning monochromator from an arc lamp, which is then plane-polarized. The differential absorption is determined directly by sequentially probing the sample with right and left circularly plane-polarized light. Sample detectability in circular dichroism is background limited since the signal represents a small change (the polarization dependent differential absorption) on top of a large background (the intrinsic absorption of the compound). For both polarimetry and circular dichroism

detection sample concentration requirements are usually in the μM range with the most favorable mass detection limits in a range above 10 ng. It is possible to determine an enantiomer excess above about 0.1 %.

5.8 POSTCOLUMN REACTION SYSTEMS

Postcolumn reaction systems are convenient for on-line derivatization used to improve sample detectability or detection selectivity for target analytes, often present in low concentrations or complex matrices [228-230]. The derivatization reaction does not have to yield a single, stable product, provided that the reaction is reproducible. Reactions must be reasonably fast (< 20 min), however, for convenient real time detection and to maintain the separation integrity, and excess reagent must not interfere with detection. Efficient mixing of the derivatizing reagent and column eluent is required and usually reaction solvents must be miscible with the mobile phase. A significant source of detector baseline noise, leading to an increase in detection limits, arises from the additional pumps and auxiliary equipment required to facilitate continuous operation of the reaction system. Dual wavelength detection with one wavelength set close to the isosbestic point for the reagent and the other close to the absorbance maximum for the analytes allows a significant reduction of pump noise for absorbance detection [231]. Although many suitable reaction chemistries have been described [228,232], the general complexity of postcolumn reaction systems and the need for lengthy optimization restricts their use for occasional sample problems. The main applications are for the detection of amino acids and biogenic amines [233], carbohydrates [234,235], pesticides and herbicides [236-238] and metal ions [168,179] in clinical, environmental and food samples.

The postcolumn reaction system must provide for the continuous addition of controlled volumes of one or more reagents to the column eluent, followed by mixing of the eluent-reagent mixture and incubation for some time and temperature governed by the needs of the reaction. Detection of the reaction products is usually by absorption, fluorescence or electrochemical techniques. Some typical postcolumn reaction configurations are shown in Figure 5.23. A piston or pneumatic pump is used to deliver the reagent to the reactor at a constant low-pulse flow rate to facilitate reproducible reaction conditions and a stable detector baseline. Peristaltic pumps are suitable for use with segmented flow systems that generally operate at low pressures. Nearly all reaction detectors contain a mixing device, T- or Y-piece, or cyclone, for contacting and homogeneously mixing the column eluent and reagent streams [228,239]. The mixing device should have a small volume to minimize band broadening and to allow low reagent flow rates to reduce peak dilution. Cyclone mixers are superior in this respect as they can be constructed with a smaller dead volume ($\approx 0.1 \mu\text{l}$) and work efficiently with reagent flow rates as low as 4% of the column flow rate. The reaction vessel is usually an open tube or packed bed of appropriate dimensions to store the combined eluent and reagent volumes for the time required to obtain optimum

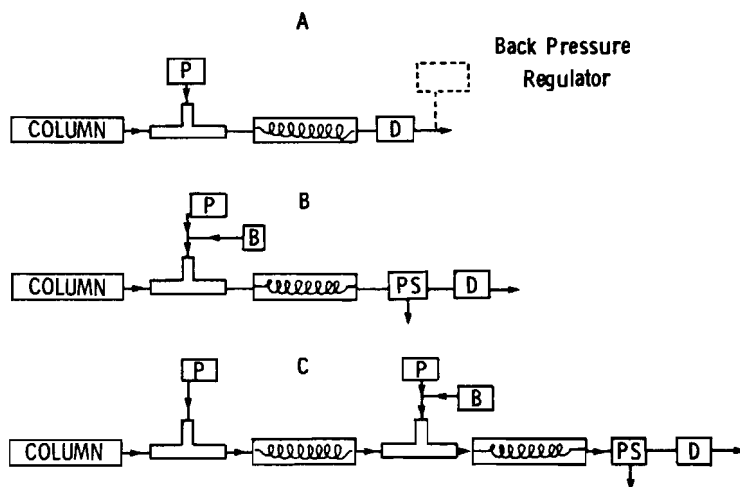


Figure 5.23. Schematic diagram of some typical postcolumn reaction configurations for liquid chromatography. A, non-segmented tubular reactor; B, segmented tubular reactor; C, extraction segmented reaction detector. P = pump, PS = phase separator, B = device for introducing bubbles and D = detector.

reaction yields. If the segmentation principle is employed, a device for introducing a bubble of liquid or gas into the eluent stream is required [228,240,241]. This results in the separation or segmentation of the column eluent into a series of reaction compartments whose volume is governed by the dimensions of the transfer tube and the frequency of bubble introduction. Prior to detection a phase separator is needed to remove the segmentation agent. Two different principles are used for phase separation. A simple T-piece with one upward opening will remove the gas from the liquid flow using density differences. In general the same principle is used for the construction of phase separators for liquid segmented systems. Here the different wettability of glass and organic polymeric tubing is used to separate aqueous and organic segments. Alternatively, the differential permeability of membranes for organic liquids and water provides a suitable mechanism for phase separation. A phase separator is also required when ion-pair formation or extraction is employed in the reaction system. Dispersion in the phase separator is often a significant source of band broadening. For reactions using solid-phase reagents, immobilized enzymes, etc., a packed bed tubular reactor is used [228,242,243]. Reactions that are too slow at room temperature are accelerated by thermostating the reaction vessel to a higher temperature. A cooling coil may be required before the detector to prevent interference in the detector operation or to avoid bubble formation.

The parabolic flow profile in an open-tubular reactor restricts applications to fast reactions if extensive band broadening is to be avoided [244]. The reaction time can be extended by using reactors prepared from optimally deformed capillary tubes [245-247]. The production of secondary flow, even at low flow rates, breaks up the parabolic

profile and minimizes band broadening. The easiest way to induce secondary flow is by using knitted or stitched open-tubular reactors. Knitted open tubular (KOT) reactors are generally fabricated from PTFE capillary tubes deformed in such a way as to produce alternating right and left loops with small coiling diameters. Each subsequent loop is bent out of the plane of its nearest neighbor. Stitched open tubular (SOT) reactors are usually prepared from narrow-bore stainless-steel capillary tubes woven through a steel mesh in serpentine fashion with alternate loops displaced to the right and left. The continuous change in the coiling direction and the bend of the coil out of plane leads to a continuous change in the direction of the secondary flow producing a plug like flow profile with almost identical velocities at the wall and tube center. The pressure drop across a KOT reactor is about twice that of an open tube, which is the limiting factor in establishing the available reaction time. For PTFE capillaries with a pressure limit of about 10 atmospheres reaction times approaching 10 minutes are possible for flow rates typical of conventional packed columns.

The time required for the reaction most profoundly influences the reactor design. For fast reactions (i.e. < 1 min) open tubular or KOT reactors are commonly used. They simply consist of a mixing device and a coiled stainless steel or PTFE capillary tube enclosed in a thermostat. The length and internal diameter of the capillary tube and the combined column and reagent flow rate through the reactor control the reaction time. Reagents such as fluorescamine and o-phthalaldehyde are frequently used in this type of system to determine primary amines, amino acids and indoles in biological and environmental samples. A common method for determining N-methylcarbamates in environmental and food extracts employs a two-stage postcolumn reaction system [236,237]. In the first reactor N-methylcarbamates are rapidly hydrolyzed to methylamine which is then converted in the second reactor to a highly fluorescent isoindole by reaction with o-phthalaldehyde and 2-mercaptoethanol.

For reactions of intermediate kinetics (i.e. reaction times < 5 and > 1 min) a KOT or packed bed reactor is used. The packed bed reactor is constructed from a length of column tubing packed with an inert material of small diameter, such as glass beads. The pressure drop across the packed bed reactor limits the length and smallest particle size that can be used. The inner diameter of the reactor (e.g. 2.5-6.0 mm) can be varied to adjust the reactor volume to the required reaction time. The packed bed reactor is particularly useful for reactions involving solid-phase reagents such as catalysts, immobilized enzymes and metallic reducing agents [228,229,242,243]. Since no reagent solutions are added in this case, there are no additional pumps or mixing units required and no dilution or mixing problems associated with postcolumn reagent addition.

For slow reactions (reaction times > 5 min) an air or liquid segmented reactor is typically used. Segmented systems are also required for reactions that require separation of the reagent from the reaction product by solvent extraction, for example when ion-pair reagents are used to form the product for detection. The reaction products are usually of different polarity to the reagent, permitting their separation by extraction

in the continuous flow mode by segmenting with immiscible organic solvent plugs (the extraction solvent) or by air segmentation with an additional flow of extraction solvent.

5.8.1 Photoreactors

Photolysis may be used to convert some analytes into easier to detect products or products suitable for subsequent on-line chemical derivatization [137,248]. Photoreactors are simple in design consisting of one or more high-powered discharge lamps in a reflective housing with fans or a Peltier cooler for heat removal. A typical arrangement uses a cylindrical tube mercury or xenon discharge lamp around which is wrapped a PTFE knitted open-tubular (KOT) capillary reactor. PTFE has excellent transparency to UV light and is preferable to quartz for fabrication of the reaction coil. For the low flow rates typical of small-bore columns a flow through reaction cell made from a length of fused-silica capillary tube with a window created by removing a section of the protective polyimide coating is generally used [249]. A selectable-power photoreactor was described that contains a central lamp around which is wrapped the reaction coil with four other lamps arranged at perpendicular axes to the reaction coil in a reflective housing [250]. By switching on various lamp combinations the light intensity can be optimized for the reaction while minimizing undesirable photodecomposition or other secondary reactions resulting in a lower yield of the detection product. The parameters that affect the extent of reaction include the residence time in the reaction coil (column flow rate and coil dimensions), mobile phase composition (solvents, pH, oxygen concentration, buffer salts) and the irradiation wavelength and its intensity [237,248,251]. Photolysis can result in the formation of products with a different spectra to the original analyte. Thus, either full spectra or individual wavelength absorption ratios for lamp on and lamp off conditions were suggested for confirmation of analyte identity [252,253]. The high specificity of the photochemical reaction detector is useful for particular applications but the number of compounds that yield useful detection products is limited [248].

5.9 INDIRECT DETECTION

Indirect detection is an alternative to derivatization for the detection of analytes with a weak detector response. It is commonly used in ion exchange (particularly ion chromatography) and ion-pair chromatography with absorbance, fluorescence or amperometric detection [168,254,255]. This requires the selection of an eluent ion with favorable detection properties to regulate the separation process and provide a constant detector signal. Detector transparent analyte ions cause displacement of eluent ions from the eluted band and a decrease in the detector response compared with the steady state signal for the mobile phase. The detected ion concentration is coupled to the retention mechanism, which can result in the appearance of additional system peaks in the chromatogram (section 4.3.3.2). These applications should be

distinguished from vacancy chromatography, in which the detector response is simply a difference chromatogram without involving a displacement mechanism [256]. Indirect detection techniques also include postcolumn response enhancement or diminution by inclusion complexation, quenching or hydrophobic adsorption of (usually) a detectable fluorescent additive and ion replacement or enzyme reactions to generate detectable species [257-259]. These techniques are not further discussed here except to note that vacancy chromatography generally provides poor sample detectability and the other methods are restricted to a limited number of analytes with specific properties.

Indirect detection provides a universal detection method for analytes sharing the same retention mechanism. The concentration limit of detection is given by $C_{LOD} = C_E / (TR \times DR)$ where C_E is the concentration of the detectable ion in the eluent. The other terms that influence the detector response are the transfer ratio (ratio of the number of molecules of the mobile phase additive displaced by each analyte molecule), TR, and the dynamic reserve (the signal-to-noise ratio of the background signal), DR. The relatively high absorption of the eluent ion in indirect UV detection causes an increase in the detector baseline noise. This noise limits analyte detectability. The UV-transparent analytes at low concentration provide a small change in the high background signal, which must be distinguished from baseline noise. For a reasonable linear response range and favorable sample detectability the concentration of UV absorbing eluent ion should be low enough that Beer's law is still valid, while at the same time the concentration must be appropriate for the sample separation conditions. All other things being equal, the concentration of the absorbing eluent ion should be as small as possible and the transfer ratio and dynamic reserve as large as possible. All three properties are interrelated and an optimum detector response may require simultaneous optimization of the chromatographic system. In general, detection limits for indirect UV detection of typical ions separated by ion chromatography are comparable to conductivity detection but not better than conductivity detection.

5.10 REFERENCES

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6.1 INTRODUCTION

Thin-layer chromatography (TLC) can trace its origins to the introduction of drop chromatography in the late 1930s [1-4]. A microscope slide was covered with a layer of aluminum oxide, on which one drop of extract was spotted, followed by dropwise addition of solvent onto this spot. Separated substances were visualized as circular zones radiating from the original spot center. For analytical applications drop chromatography offered a faster, more convenient and more powerful separation tool than conventional column chromatography as originally described by Tswett. Thin-layer chromatography as we know it today was not established until the 1950s, in large part due to the efforts of Kirchner and Stahl [5,6]. These workers devised standardized procedures to improve the separation performance and reproducibility of thin-layer chromatography, paving the way for its commercialization, as well as contributing many new applications. The 1970s saw the introduction of fine particle layers and associated instrumentation for their correct use [7-9]. In this form thin-layer chromatography became known as high performance TLC, instrumental TLC, or modern TLC to distinguish it from its parent, now generally referred to as conventional TLC. Modern TLC did not displace conventional TLC from laboratory studies and the two approaches coexist today because of their complementary features. Conventional thin-layer chromatography can provide a quick, inexpensive, flexible and portable method for monitoring synthetic reactions and similar applications. Virtually no instrumentation is required and the method can be learned quickly. On the other hand, modern thin-layer chromatography is characterized by the use of more expensive fine particle layers for faster and more efficient separations, and requires the use of instruments for convenient (automated) sample application, development and detection [1,9-11]. Conventional thin-layer chromatography is considered a qualitative or semi-quantitative method while modern thin-layer chromatography provides accurate and precise quantitative results based on *in situ* measurements and a record of the separation in the form of a chromatogram. Since the 1970s modern thin-layer chromatography has evolved by technical improvements to layers, instrumentation and increased automation facilitated by computer-controlled devices. Major innovations include automated multiple development, interfaces to

spectroscopic instruments (sections 9.2.2.5 and 9.3.1.4) and emerging techniques for video densitometry.

Thin-layer chromatography is a type of liquid chromatography in which the stationary phase is in the form of a thin layer on a flat surface rather than packed into a tube (column). It is a member of a family of techniques that include some types of electrophoresis and paper chromatography more generally referred to as planar chromatography. Since we will not discuss electrophoresis here, and since thin-layer chromatography has virtually superseded paper chromatography, we will confine ourselves to a discussion of thin-layer chromatography.

In the basic experiment, the sample is applied to the layer as a spot or band near to the bottom edge of the layer. The separation is carried out in a closed chamber by either of two mechanisms. By contacting the bottom edge of the layer with the mobile phase, which advances through the layer by capillary forces, or by forcing the mobile phase through the layer at a controlled velocity by applying external pressure or other means. A separation of the sample results from the different rates of migration of the sample components in the direction traveled by the mobile phase. After development and evaporation of the mobile phase, the sample components are separated in space; their position and quantity being determined by visual evaluation or densitometry.

6.2 ATTRIBUTES OF LAYERS AND COLUMNS

Separations by column liquid chromatography (HPLC) and thin-layer chromatography occur essentially by the same physical processes. The two methods are often considered competitors when it would be more realistic to consider them complementary techniques. The favorable attributes of thin-layer chromatography providing for its co-existence alongside column liquid chromatography are summarized in Table 6.1 [1].

The elution mode is commonly used for column liquid chromatography and the development mode for thin-layer chromatography. Each sample component must travel the complete length of the column in column liquid chromatography and the total separation time is determined by the time required for the slowest moving component to reach the detector. While for thin-layer chromatography the total time for the separation is the time required for the solvent front to migrate a predetermined distance, and is independent of the migration distance of the sample components. Excessively retained components extend the separation time in column liquid chromatography while components accumulated at the head of the column are completely eluted. If this is not possible then permanent alteration of the properties of the column may occur, eventually making the column useless for the separation. Thin-layer chromatography plates are disposed of at the conclusion of each separation and are immune from this problem. Since the total sample occupies the chromatogram in thin-layer chromatography the integrity of the analysis is guaranteed but can only be implied in column chromatography.

Table 6.1

Attributes of thin-layer chromatography

Attribute	Application
Separation of samples in parallel	<ul style="list-style-type: none"> ● Low-cost analysis and high-throughput screening of samples requiring minimal sample preparation.
Disposable stationary phase	<ul style="list-style-type: none"> ● Analysis of crude samples (minimizing sample preparation requirements) ● Analysis of a single or small number of samples when their composition and/or matrix properties are unknown ● Analysis of samples containing components that remain sorbed to the separation medium or contain suspended microparticles
Static detection	<ul style="list-style-type: none"> ● Samples requiring postchromatographic treatment for detection ● Samples requiring sequential detection techniques (free of time constraints) for identification or confirmation
Storage device	<ul style="list-style-type: none"> ● Separations can be archived ● Separations can be evaluated in different locations or at different times ● Convenient fraction collection for multimodal column/layer chromatography
Sample integrity	<ul style="list-style-type: none"> ● Total sample occupies the chromatogram not just that portion of the sample that elutes from the column.

In addition, the use of the elution mode in column liquid chromatography means that separations must be performed sequentially. The time required to analyze a group of samples is equal to the time to separate one sample, including column re-equilibration time, multiplied by the number of samples in the group. Thin-layer chromatography allows samples to be separated in parallel with the potential for a significant reduction in the total separation time. Depending on the plate size, between 18 and 72 samples and standards can be separated simultaneously in the same time required to separate a single sample. Some of this advantage is lost because it is easier to automate column liquid chromatography, and unattended overnight operation is possible. Whereas for thin-layer chromatography the individual steps of sample application, development and detection can be automated, but manual intervention is required to move the plate from station to station. For thin-layer chromatography, it is also necessary to include the time for sample application and scanning of the plate. Even after making suitable allowances for these points, thin-layer chromatography generally provides a significant reduction in separation time.

At the end of the separation in thin-layer chromatography, the mobile phase is evaporated and the separation becomes immobilized. Detection, therefore, takes place in the presence of the stationary phase, is independent of time constraints, and all separated zones are simultaneously accessible. This provides flexibility in the choice of detection strategies and even the possibility of archiving separations for evaluation by different detection processes applied at different times or locations. Several detection techniques can be applied sequentially, as long as they are nondestructive. For optical detection, the minimum detectable quantities are similar for both column liquid and thin-layer

chromatography with, perhaps, a slight advantage for column liquid chromatography. Direct comparisons are difficult because of the differences in detection variables and how these are optimized. Detection in thin-layer chromatography, however, is generally limited to optical detection without the equivalent of refractive index and electrochemical detection available for column liquid chromatography. The ease of postchromatographic reactions used to enlarge the application range of optical detection in thin-layer chromatography largely offsets this disadvantage.

In the light of the above discussion, thin-layer chromatography is most effective for the low-cost analysis of simple mixtures, especially when a large number of samples require analysis. For the rapid analysis of samples requiring minimum sample cleanup or where thin-layer chromatography allows a reduction in the number of sample preparation steps (e.g. the analysis of samples containing components that remain sorbed to the stationary phase or contain suspended microparticles). For the analysis of substances with poor detection characteristics requiring postchromatographic treatment for detection. In addition, it is the most suitable technique for determining general sample composition, since all sample components are located in the chromatogram.

Traditionally, thin-layer chromatography has been used in large-scale surveillance programs. These include the identification of drugs of abuse and toxic substances in biological fluids in forensic toxicology [12-15]. The identification of unacceptable levels of residues from drugs used to prevent disease or promote growth in farm animals [16-19]. To ensure a safe water supply by monitoring crop-protecting agents used in modern agriculture [20,21]. To ensure conformity with the label declaration of pharmaceutical products [22]. In these applications, thin-layer chromatography is often used with other methods in a pyramid strategy. This strategy employs a screening step (thin-layer chromatography) to identify suspect samples and a confirmation step (the most suitable analytical method) to establish the contaminant level in suspect samples. The benefits of the pyramid approach are lower costs and an increase in the number of samples processed to identify violated samples [1]. Thin-layer chromatography is selected for the screening step because: (1) single use of the stationary phase minimizes sample preparation requirements; (2) parallel separations enhance sample throughput; (3) ease of postchromatographic derivatization improves selectivity and specificity for the analysis; and (4) several screening protocols for different analytes can be carried out simultaneously.

Thin-layer chromatography remains one of the main methods for class fractionation and speciation of lipids [23,24] and is used increasingly to determine the botanical origin, potency, and flavor potential of herbs and spices [25-27]. In the pharmaceutical industry, it is used for the analysis of complex and dirty samples with poor detection characteristics and for stability and content uniformity testing [28-31]. It continues to be widely used in the standardization of plant materials used as traditional and modern medicines. In addition, it retains an historic link with the characterization of dyes and inks and the control of impurities in industrial chemicals.

In other cases, column liquid chromatography is the preferred method of separation, particularly if a large plate number is required for the separation or the sample prepa-

ration time is long compared with the separation time. Separations by size-exclusion and ion-exchange chromatography are usually easier by column liquid chromatography. Since there are few suitable layers for the separation of biopolymers by thin-layer chromatography, column liquid chromatography and electrophoresis are commonly used for these applications. Finally, column liquid chromatography is favored for trace analysis using selective detectors unavailable to thin-layer chromatography.

6.3 THEORETICAL CONSIDERATIONS

A unique feature of thin-layer chromatography is the presence of a third phase, the vapor phase, in contact with the mobile and stationary phases. Alterations in the composition and velocity of the mobile phase moving through the layer induced by the vapor phase are possible in ways that are not observed for column liquid chromatography. Also, the mobile phase normally penetrates a dry layer in thin-layer chromatography by capillary action. The flow of solvent at the developing front is generally unsaturated and the speed with which the front moves is dependent on experimental and environmental conditions [32-35]. Capillary forces are stronger in the narrow interparticle channels, leading to a more rapid advance of the mobile phase. Larger pores below the solvent front are filled at a slower rate resulting in an increased thickness of the mobile phase layer. If the vapor phase and mobile phase are not in equilibrium, evaporation will cause a loss of mobile phase from the layer surface and a decrease in the solvent front velocity. On the other hand, the dry layer ahead of the solvent front progressively adsorbs vapor, filling some of the pores and interparticle channels, and increasing the apparent velocity with which the solvent front migrates. During the chromatographic process a solvent composition gradient is produced as the mobile phase moves through the sorbent due to selective adsorption by the stationary phase of the solvent component with the higher affinity for the stationary phase. Gaining satisfactory control over the above processes in large volume chambers is almost impossible. Various kinds of sandwich chambers, which either eliminate or minimize contact of the plate surface with the vapor phase, offer reasonable control of the mobile phase velocity.

6.3.1 Retardation Factor

The fundamental parameter used to characterize the position of a sample zone in a thin-layer chromatogram is the retardation factor, or R_F value. It represents the ratio of the distance migrated by the sample compared to the distance traveled by the solvent front, and for linear development is given by Eq. (6.1)

$$R_F = Z_X / (Z_f - Z_o) \quad (6.1)$$

where Z_X is the distance traveled by the sample from its origin, $(Z_f - Z_o)$ the distance traveled by the mobile phase from the sample origin, Z_f the distance traveled by the

solvent front measured from the mobile phase entry position (the solvent level at the start of the separation), and Z_0 the distance from the sample origin to the position used as the origin for the mobile phase. The boundary conditions for R_F values are $1 \geq R_F \geq 0$. When $R_F = 0$, the spot does not migrate from the origin, and for $R_F = 1$, the spot is unretained by the stationary phase and migrates with the solvent front. Although R_F values are widely quoted, they are difficult to determine accurately [32,36]. Systematic errors result from the difficulty in locating the exact position of the solvent front. If the adsorbent layer, mobile phase and vapor phase are not in equilibrium then condensation of the vapor phase or evaporation of the mobile phase in the region of the solvent front will give an erroneous R_F value. Evaporation of mobile phase from the surface of the layer in unsaturated chambers generally results in higher R_F values. The reproducibility of R_F values in unsaturated chambers may be poor and concave solvent fronts formed resulting in higher R_F values for samples nearest to the edges of the layer. The thermodynamic solvent front, which may be slightly lower than the visible solvent under conditions of unsaturated flow, can be determined by using an unretained substance as a solvent-front marker [37]. Not only does the level of saturation influence the reproducibility of R_F values but also can result in changes in the migration order [36]. To improve the reproducibility of R_F values determined at different times or locations, the saturation grade of the developing chamber is used to interconvert observed R_F values between different chamber designs [36]. When the analytes are available as standards, it is common practice to separate the standards and samples in the same system for identification purposes. In surveillance programs, the simultaneous separation of appropriate standard substances is used to improve the certainty of identification by correcting observed R_F values to standard R_F values for automated library searches [12-15]. Using the mean list method, for example, all substances that migrate in a R_F window that might be confused, are ranked and compared across a number of separation systems. If the separation systems are complementary, the list of possible substances that might be confused will become shorter as an increasing number of substances fall outside the identification window for the unknown. Eventually only a handful of possible substances remain on the list. At which point suitable selective separation and spectroscopic techniques are used to confirm the identification of the unknown.

R_F values are generally calculated to two decimal places. Some authors prefer to tabulate values as whole numbers, as hR_F values equivalent to $100 R_F$. The R_F value is not linearly related to the distribution properties of the separation system. The R_M value is used in studies that attempt to correlate migration properties to solute structure. The R_M value is equivalent to the ratio of the residence time of the solute in the stationary and mobile phases, and is formally equivalent to the retention factor ($\log k$) in column liquid chromatography. It is calculated from the R_F value by R_M (or $\log k$) = $\log [(1 - R_F) / R_F]$.

6.3.2 Flow Through Porous Layers

The common methods of mobile phase transport through the layer are capillary action, forced flow, and electroosmosis. Ease of implementation results in capillary flow

being the dominant transport mechanism, although it is also the most restrictive. Forced flow has a number of advantages over capillary flow but requires sophisticated instruments and is less widely used. Electroosmotic flow has shown promise as a transport mechanism, but there is confusion over conditions required for its utilization. For now, it remains a curiosity in need of further development.

6.3.2.1 Capillary Flow

The force driving mobile phase migration in thin-layer chromatography is the decrease in the free energy of the solvent as it enters the porous structure of the layer; the transport mechanism is a result of capillary action [32,33]. It is an empirical fact that in the absence of a significant exchange of solvent flux with the vapor phase the position of the solvent front with respect to time is adequately represented by the simple quadratic expression $(Z_f)^2 = \kappa t$, and after differentiation, the velocity of the solvent front by $u_f = \kappa / 2Z_f$. Where Z_f is the distance of the solvent front position above the solvent level in the developing chamber, κ the velocity constant (cm^2 / s), t the time from contacting the layer with the solvent, and u_f the solvent front velocity. In the absence of equilibrium, rather complex correction factors must be applied to the quadratic equation relating Z_f to the development time [32,33]. The undesirable feature of capillary flow, namely, decreasing mobile phase velocity with increasing migration distance, is apparent from the form of the solvent-front velocity expression. The outcome is longer separation times and a reduced separation potential.

The velocity constant, κ , is related to the experimental conditions by equation (6.2)

$$\kappa = 2 K_o d_p (\gamma / \eta) \cos \theta \quad (6.2)$$

where K_o is the permeability constant of the layer, d_p the average particle diameter, γ the surface tension of the mobile phase, η the viscosity of the mobile phase, and θ the contact angle. The permeability constant is a dimensionless constant which takes into account the profile of the external pore size distribution, the effect of porosity on the permeability of the layer, and the ratio of the bulk liquid velocity to the solvent front velocity. Experimental values for K_o vary for different layers. Typical values for precoated layers, however, fall into the range 0.001 to 0.002 and are similar to values for slurry-packed columns [38].

Equation (6.2) indicates that the velocity constant should increase linearly with the average particle size resulting in a solvent-front velocity that should be larger for coarse-particle layers than for fine-particle layers. Also, from Eq. (6.2) we see that the velocity constant varies linearly with the ratio of the surface tension of the solvent to its viscosity. Thus, solvents which maximize this ratio (and not just optimize one of the parameters) are preferred for thin-layer chromatography. The contact angle for most organic solvents on silica gel and polar chemically bonded layers is generally close to zero ($\cos \theta = 1$). This is not the case for chemically bonded reversed-phase layers containing long-chain, alkyl groups. For these layers, the contact angle of the mobile phase increases rapidly with the water content and, when the water content is about 30-40%, $\cos \theta$

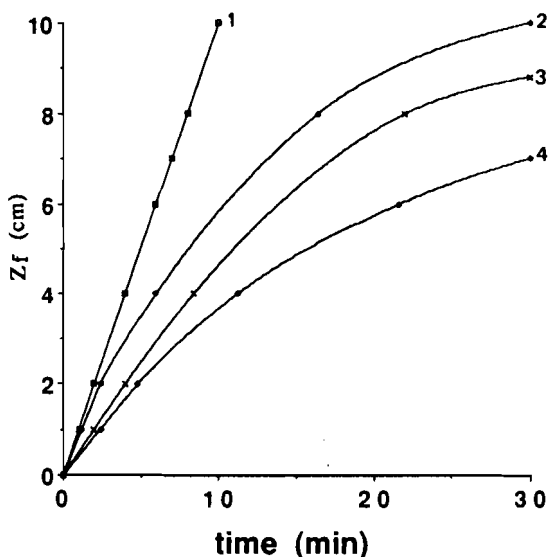


Figure 6.1. Relationship between the solvent-front position and time for a forced flow separation (1) and capillary flow separations with an exposed layer in a saturated chamber (2), a covered layer (sandwich chamber) (3), and an exposed layer in an unsaturated atmosphere (4). (From ref. [33]; ©Research Institute for Medicinal Plants).

becomes less than 0.2-0.3 [39]. Separations dependent on capillary flow are just about impossible under these conditions. For reasonable compatibility with aqueous mobile phases, hydrophobic layers with less silanization and/or larger particle size are generally used for reversed-phase thin-layer chromatography.

The above discussion is applicable to layers unperturbed by the presence of a vapor phase, such as in a sandwich-type-developing chamber. In practice, most separations are performed in large volume chambers in the presence of a vapor phase. It is almost impossible to saturate these chambers and temporal and spatial vapor equilibrium is unlikely to exist. Two opposing phenomena are expected to influence the rate of mobile phase migration. Vaporization of the mobile phase from the wetted layer is reasonably expected to depend on the wetted surface area of the plate and the vapor pressure of the solvents in the chamber. The loss of mobile phase from the layer will result in a reduction of the mobile phase velocity from that indicated by Eq. (6.2), Figure 6.1 [34,40]. When a dry layer is placed in the developing chamber, it progressively adsorbs mobile phase vapors. The pores of the dry layer ahead of the solvent front fill slowly with adsorbed vapor and the apparent porosity of the layer diminishes. Since the porosity of the layer decreases, the velocity constant increases slowly with time. The effect of vaporization is generally small if the chamber atmosphere is close to saturation. In this case, adsorption of mobile phase vapors by the dry layer will tend to dominate. Thus, the mobile phase velocity in a large-volume chamber will tend to be greater than

that given by Eq. (6.2) and increases with time, if the layer is not conditioned in the chamber atmosphere prior to the start of development.

6.3.2.2 *Forced Flow*

Forced flow enables the mobile phase velocity to be optimized without regard to the deficiencies of capillary flow systems. Rotational planar chromatography uses centrifugal force generated by spinning the layer about a central axis, to drive the mobile phase through the layer [41,42]. The mobile phase velocity is a function of the rotation speed and the rate at which the mobile phase is supplied to the layer. Since the layer is not enclosed, the ultimate velocity of the solvent front is limited by the amount of solvent that can be kept within the layer without floating over the surface. At high rotation speeds, the velocity of the solvent front is approximately constant in the linear development mode. Rotational planar chromatography is used for preparative-scale applications (section 11.2) more so than analytical separations.

An alternative approach to forced flow is to seal the layer with a flexible membrane or an optically flat, rigid surface under hydraulic pressure, and to deliver the mobile phase to the layer by a pump [9,41,43-46]. Adjusting the solvent volume delivered to the layer optimizes the mobile phase velocity. In the linear development mode, the mobile phase velocity (u_f) will be constant and the position of the solvent front (Z_f) at any time (t) after the start of development is described by $Z_f = u_f t$. The mobile phase velocity no longer depends on the contact angle and solvent selection is unrestricted for reversed-phase layers in forced flow, unlike capillary flow systems.

When a liquid is forced through a dry layer of porous particles sealed from the external atmosphere, the air displaced from the layer often forms a second front (beta front), moving behind the mobile phase-air front (alpha front) [47-49]. The beta front usually has an irregular (wavy) shape. During the liquid-air displacement process, some air is displaced instantaneously and escapes ahead of the front. The remainder is displaced at a slower rate and leaves the layer by dissolution in the mobile phase, or as microbubbles moving with the mobile phase. The solubility of air in the mobile phase depends on the applied pressure, and at some critical pressure, all of the gas will dissolve and the layer becomes completely wetted by the mobile phase. The space between the alpha and beta fronts is referred to as the disturbing zone. It can be distinguished from the completely wetted region of the layer by its apparent optical density. Solutes moving in the disturbing zone, or passed over by it, are often distorted and difficult to quantify by scanning densitometry. The disturbing zone is minimized or eliminated by predevelopment of the layer with a weak mobile phase in which the sample does not migrate. This dislodges the trapped air from the layer before starting the separation. Increasing the local pressure by using a backpressure regulator increases the solubility of air in the mobile phase and provides an alternative approach to minimize the disturbing zone.

Multiple solvent fronts are also observed for both forced flow and capillary flow systems with mobile phases containing solvents of different elution strength [32,49]. As the mobile phase moves through the layer, it becomes depleted in component with

greatest affinity for the stationary phase. Eventually a secondary front is formed that separates the equilibrium mobile phase composition from the mobile phase composition now totally depleted in the solvent selectively adsorbed by the stationary phase. For a mobile phase consisting of n solvents, as many as $n+1$ solvent fronts are possible. This process is referred to as solvent demixing and adds to the complexity of method development. Solvent demixing results in sample components moving in regions of different solvent strength and selectivity with retention that is no longer easily related to the bulk mobile phase composition. Sample zones close to a secondary front are often focused into narrow bands with a different shape to neighboring zones, which allows demixing conditions to be identified. Solvent demixing is intensified in developing chambers with an unsaturated atmosphere.

6.3.2.3 *Electroosmotic Flow*

Electroosmotic flow has emerged as a viable alternative transport mechanism to pressure-driven flow in column chromatography (section 8.4.1). Benefits include a plug-flow profile (reduced transaxial contributions to zone broadening) and a mobile phase velocity that is independent of the column length and particle size (assuming Joule heating is not a limiting factor). The simple experimental arrangement for generating electroosmotic flow and declining interest in forced flow are responsible for the current interest in electroosmotic-driven flow as a means of overcoming the limitations of capillary flow in thin-layer chromatography [11,50].

The current status of electroosmotic-driven flow in thin-layer chromatography (planar electrochromatography) is probably more confusing than reassuring, although recent studies have brought some enlightenment to this technique [50-54]. Early studies of electroosmotic flow in vertically mounted layers are now believed, for the most part, to be the result of thermal effects [51]. Enhanced flow resulting from forced evaporation of the mobile phase from a solvent-deficient region at the top of the layer. In horizontal developing chambers, as well as electroosmotic flow in the direction of migration there is also flow to the surface of the layer. Joule heating offsets this effect by evaporation of mobile phase from the layer surface. Under conditions of excessive wetting or drying of the layer, degradation of the separation quality occurs [53,54]. For acceptable separation quality in reversed-phase separations, voltage and buffer concentration should be optimized to minimize either excessive wetting or drying of the layer.

True electroosmotic flow has been demonstrated in horizontally mounted layers at modest field strengths ($< 1 \text{ kV / cm}$) with mobile phases of high dielectric constant. Still unclear is which solvents can be used, the need for prewetted layers and ions as current carriers, the effect of local heating on zone profiles, and the effect of binder chemistry on flow, mass transfer, and thermal effects. Compared with capillary flow faster separations have been demonstrated, but the influence of flow velocity on efficiency was only treated in a qualitative sense. So far no comprehensive analysis of the kinetic properties of separations under conditions of electroosmotic flow have been performed in thin-

layer chromatography, and too little information is available to warrant formulation of a theoretical model until mechanistic details are clarified.

6.3.3 Zone Broadening and the Plate Height Equation

A discussion of zone broadening in thin-layer chromatography is inherently more complex than for column liquid chromatography. The reasons for this are due to both the practice of the technique and the limitations of the subsequent theoretical treatment [11,32,55-59]. Visual detection cannot be relied upon to determine zone dimensions since the eye is incapable of accurately identifying zone boundaries at low concentration and instrumental techniques are essential. The size of developed zones in thin-layer chromatography are normally in the 2-10 mm range and sample application zones about 1-2 mm. Consequently, the size of the starting zone is never small compared to the size of developed zones. The contribution of the starting zone length to those of the separated zones can be removed by consideration of their variance. The determination of zone variance is straightforward for developed zones but presents some difficulty for the starting zone [60]. On first contacting the sample zone the advancing solvent front can cause a reshaping of the zone and, since its leading edge is probably not fully saturated, all the pores holding sample will not be filled simultaneously. Consequently, adsorbed sample may not be displaced instantaneously from the sorbent surface, and localized solvent saturation may limit the rate of solute dissolution in the mobile phase. These factors will generally prevent an accurate value for the starting zone dimensions relevant to the separation from being obtained.

Zones in modern thin-layer chromatography are generally symmetrical and their profiles recorded by scanning densitometry are Gaussian to a good approximation. The average plate height (H_{obs}) and plate number (N_{obs}) can be calculated directly from the chromatogram as

$$H_{\text{obs}} = w^2 / a Z_X \quad (6.3)$$

$$N_{\text{obs}} = a (Z_X / w)^2 \quad \text{and} \quad Z_X = R_F (Z_f - Z_o) \quad (6.4)$$

where w is a parameter describing the peak width and a is an appropriate scaling factor for a Gaussian model (section 1.5). When w is the peak width at the base a has the value 16, and when w is the peak width at half height, a is 5.54. In thin-layer chromatography all separated zones migrate different distances, which is not the case for column chromatography. Consequently, separated zones only experience those theoretical plates through which they travel and, therefore, the average plate height and plate number depend on the zone migration distance. Also, an average value is used to indicate that the mobile phase velocity for capillary flow is variable throughout the chromatogram and individual zones migrate through regions of different local efficiency. Thus, the average plate height and the observed plate height in the chromatogram are identical. For the same reason, the average plate height depends

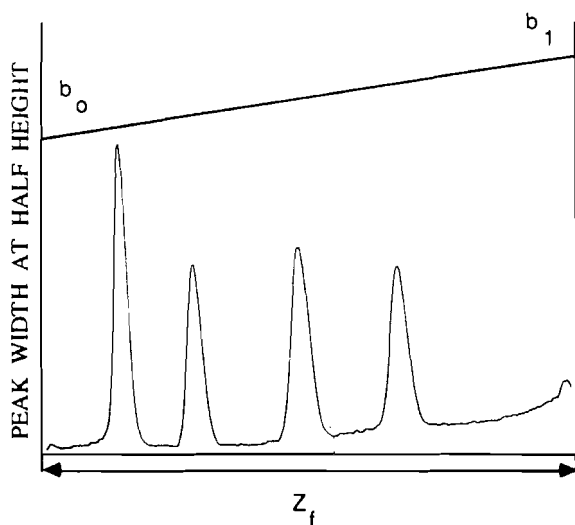


Figure 6.2. Change in peak width at half height as a function of migration distance for a test mixture on a high performance layer. Linear regression analysis is used to obtain values for b_0 and b_1 .

on the distance between the sample application position and the mobile phase entry position or solvent level for the layer (Z_0). As Z_0 increases, sample zones are forced to migrate with a lower mobile phase velocity resulting in additional zone broadening [59].

Kaiser proposed an alternative method to determine the plate height based on linear extrapolation of the peak width at half height (given the symbol b) as a function of ($Z_f - Z_0$) [7,57]. The hypothetical expected peak widths at half height corresponding to $R_F = 0$ (b_0) and $R_F = 1$ (b_1) are obtained as indicated in Figure 6.2. The real plate number (N_{real}) and the real plate height (H_{real}) can then be defined according to equations (6.5) and (6.6). This approach recognizes the importance of the starting zone dimension on the plate height value and provides a simple means to calculate plate height values for any R_F value independent of the location of substances in the chromatogram. For the purposes of standardization N_{real} and H_{real} are usually quoted for $R_F = 0.5$ or 1.0.

$$N_{\text{real}} = 5.54 [Z_X / (b_X - b_0)]^2 \quad (6.5)$$

$$H_{\text{real}} = (b_X - b_0)^2 / 5.54 Z_X \quad (6.6)$$

Ideally the rest diffusion value, b_0 , should be small compared to b_1 to maximize N_{real} . In practice the extrapolated value for b_0 is somewhat independent of the size of the sample application zone for normal zone sizes and depends significantly on the quality of the layer. The rest diffusion value is probably not an accurate estimate of the starting zone size at the time of initial migration [59].

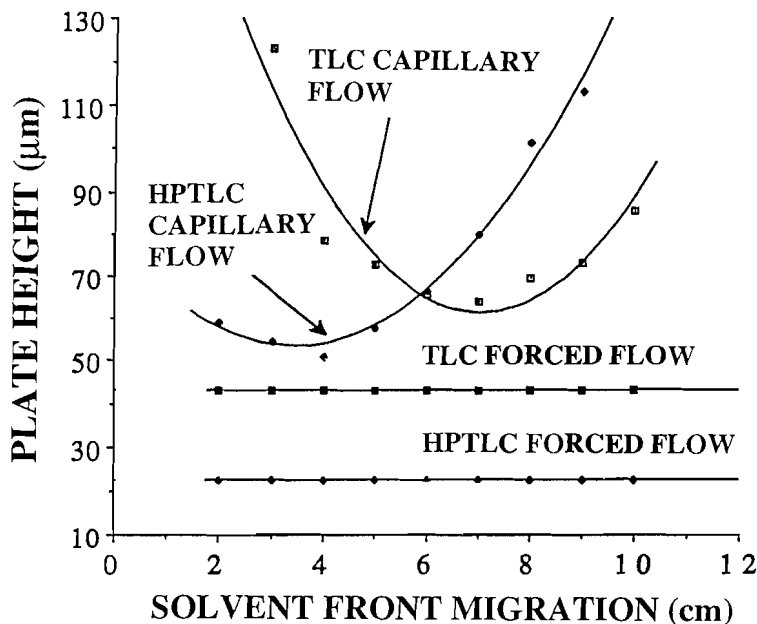


Figure 6.3. Variation of the average plate height as a function of the solvent-front migration distance for conventional and high performance layers using capillary flow and forced flow at the optimum mobile phase velocity. (From ref. [61]; ©Elsevier).

Capillary flow results in a mobile phase velocity that is spatially dependent (declines with the solvent-front migration distance) with a maximum velocity at any position on the layer that is less than the velocity required for optimum performance [11,59,61]. For common solvents the mobile phase velocity is typically in the range 0.02 to 0.005 cm/s while the optimum mobile phase velocity for high performance layers is about 0.05 cm/s [62]. A plot of the average plate height as a function of the solvent-front migration distance, Figure 6.3, reveals several important features of zone broadening with capillary flow. There is a dominant relationship between the solvent-front migration distance, the average particle size for the layer, and the separation performance. High performance layers of small particle size afford compact zones if the solvent-front migration distance does not exceed about 5-6 cm. When solvent-front migration distances are longer than this, layers of larger average particle size (conventional layers) are more efficient. This contrary finding is easily explained by the relative permeability of the layers. The mobile phase velocity for the high performance layer declines rapidly with the solvent-front migration distance until eventually zone broadening exceeds the rate of migration of the zone center. It is futile to use longer solvent-front migration distances than 5-6 cm for high performance layers for this reason. For the more permeable conventional layers the mobile phase velocity is higher for longer distances. As expected, the minimum in the plate height is shifted to longer

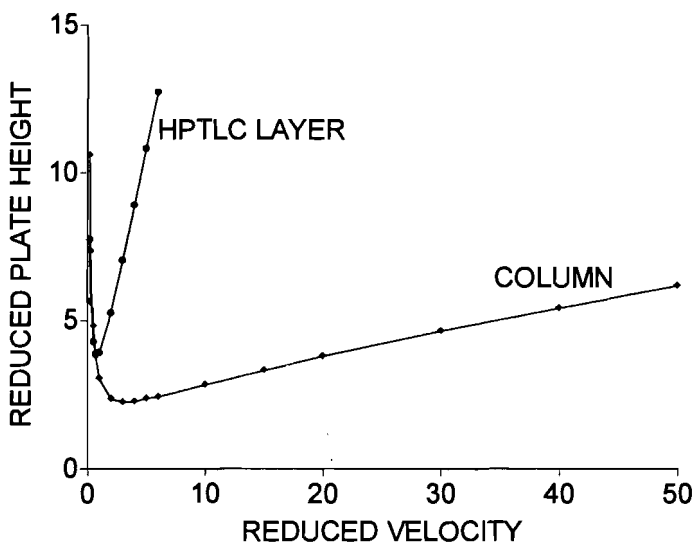


Figure 6.4. Plot of the reduced plate height against the reduced mobile phase velocity for a high performance layer and column. (From ref. [1]; ©Elsevier)

solvent-front migration distances for the conventional layer, but the intrinsic efficiency, measured by the minimum plate height, is more favorable for the high performance layer. When the development distance is optimized the separation performance of conventional and high performance layers are not very different. The virtues of the high performance layers are that it requires a shorter migration distance to achieve a given efficiency resulting in faster separations and more compact zones, which are easier to detect by scanning densitometry.

For forced flow separations a constant plate height independent of the solvent-front migration distance is obtained, Figure 6.3. The minimum plate height for capillary flow is always greater than the minimum for forced flow. This is an indication that the limited range of capillary flow velocities is inadequate to realize the optimum kinetic performance for the layers. At the mobile phase optimum velocity, forced flow affords more compact zones and shorter separation times compared with capillary flow. As expected the intrinsic efficiency increases with a reduction of the average particle size for the layer.

The variation of the reduced plate height (section 1.5.3) as a function of the reduced mobile phase velocity for a packed column and high performance layer is illustrated in Figure 6.4 [1,62]. The optimum reduced mobile phase velocity is shifted to a lower value compared with the column and the minimum in the reduced plate height (≈ 3.5) is higher than typical values for a good column (≈ 2.0 - 2.5). Also, at higher reduced mobile phase velocities the reduced plate height for the layer is significantly larger than for the column. Resistance to mass transfer is significantly higher for the layer than for a good

Table 6.2

Performance characteristics of forced flow thin-layer chromatography

Assumptions: viscosity = 3.5×10^{-4} N.s/m² and solute diffusion coefficient = 2.5×10^{-9} m²/s. (h = reduced plate height, v = reduced mobile phase velocity, ϕ = flow resistance parameter, and d_p = average particle size)

Development time (min)	Pressure drop (atm)	N_{\max}	Development length (cm)
<i>HPTLC (optimum conditions $h = 3.75$, $v = 0.8$, $\phi = 800$ and $d_p = 6 \mu\text{m}$)</i>			
4	2.1	3,550	8
9	4.7	8,000	18
25	12.9	22,200	50
50	25.8	44,400	100
<i>HPTLC (fast development option $h = 9$ and $v = 5$)</i>			
0.6	12.9	1,480	8
1.4	29.1	3,330	18
4.0	80.7	9,250	50
8.0	161.0	18,500	100
<i>HPTLC ($d_p = 3 \mu\text{m}$; other parameters as in (a))</i>			
2.0	16.5	7,610	8
4.5	37.2	17,100	18
12.5	103	47,600	50
25.0	207	95,200	100
<i>Conventional TLC ($h = 4.5$ and $v = 0.8$, $\phi = 600$ and $d_p = 9 \mu\text{m}$)</i>			
6	0.44	1,980	8
13.5	1.03	4,450	18
37.5	2.9	12,350	50
75	5.7	24,700	100

column. Consequently, separations by forced flow will be slower than those achieved with typical columns, and fast separations at high flow rates will be less efficient.

Some expectations for forced flow separations are summarized in Table 6.2 [1,50]. For a normal development distance of 18 cm a modest increase in performance (a maximum of 8000 theoretical plates) in a credible time of 9 minutes is achieved compared with typical results for capillary flow (< 5000 theoretical plates in about 25-45 min). Really significant increases in efficiency are achieved only by the use of longer layers at the expense of separation time. This can be achieved by the serial coupling of conventional size layers arranged in a stack with a special tail-to-head vertical connection between layers [46,63,64]. The optimum mobile phase velocity is sufficiently low that the pressure drop remains modest compared with typical coupled column systems for liquid chromatography. Fast separations on high performance layers, result in low efficiency, and high pressures are required if long development distances are used. These conditions are not useful for most separations, especially because layers enable separations to be performed in parallel, so the relative time per separation is not adversely impaired compared with those of other separation techniques. If a relatively large plate number is to be achieved then high pressures are required. Reducing the particle size from an average of $5 \mu\text{m}$ to $3 \mu\text{m}$ result in improved efficiency and favorable separation times, but is more demanding in terms of operating

Table 6.3

Characteristic properties of silica gel precoated layers and columns

Parameter		Layers		Column
		High performance	Conventional	High performance
Porosity	Total	0.65-0.70	0.65-0.75	0.8-0.9
	Interparticle	0.35-0.45	0.35-0.45	0.4-0.5
	Intraparticle	0.28	0.28	0.4-0.5
Flow resistance parameter		875-1500	600-1200	500-1000
Apparent particle size (μm)		5-7	8-10	d_p
Minimum plate height (μm)		22-25	35-45	2-3 d_p
Optimum velocity (mm/s)		0.3-0.5	0.2-0.5	2
Minimum reduced plate height		3.5-4.5	3.5-4.5	1.5-3
Optimum reduced velocity		0.7-1.0	0.6-1.2	3-5
Knox equation coefficients				
Flow anisotropy (A)		0.4-0.8	1.7-2.8	0.5-1.0
Longitudinal diffusion (B)		1.2-1.6	1.2-2.0	1-4
Resistance to mass transfer (C)		1.4-2.4	0.70-0.85	0.05
Separation impedance		10,000-20,000	11,000-13,000	2000-9000
Mean pore diameter (Si 60) (nm)		5.9-7.0	6.1-7.0	

pressure. There are no expected benefits from the use of conventional layers for forced flow separations.

6.3.4 Kinetic Properties of Precoated Layers

Layers have a heterogeneous structure consisting of the sorbent particles, held together by added binder, with other possible additives, such as a fluorescence indicator for visualization of UV-absorbing compounds. Typical values for the kinetic properties precoated silica gel layers are summarized in Table 6.3 [11,38,61,62,65,66]. The similar values for the interparticle porosity of columns and layers suggests that their packing density is similar, whereas the significant difference in the total porosity and intraparticle porosity indicates that the intraparticle volume of the layers is substantially smaller. A significant amount of the binder used in stabilizing the layers must be contained within the pores [38]. The mean pore size and pore size distribution of the precoated layers determined by size-exclusion chromatography are in accordance with expected values for the silica gel, tending to confirm that the binder resides largely within the pores and does not specifically block the pore entrances [65]. The difference between the flow resistance parameter of the high performance and conventional layers might arise from the presence of a greater proportion of fine particles in the high performance layer compared with the conventional layer, reflecting the fact that high performance layers are prepared from particles of a smaller average particle size initially. Layers with a narrower particle size distribution but similar average particle size could lead to a further improvement in capillary flow separations on high performance layers. The small difference between apparent average particle sizes for high performance and conventional layers is the origin of the small difference between

the minimum plate height for modern layers. Historically, conventional layers afforded poorer separations because they were prepared from sorbents with a significantly wider particle size distribution. Contemporary conventional layers are prepared from particles of smaller average size and narrower particle size distribution compared with a decade ago, reflected in their improved separation performance.

The reasons for the difference in kinetic performance between columns and layers can be deduced from the coefficients of the Knox equation, Table 6.3. The A coefficient is a measure of flow anisotropy within the streaming part of the mobile phase and is related to the packing density and homogeneity of the layer. For high performance layers the A coefficients are smaller than those for typical columns, indicating that the layers are homogeneously packed and have a good packing structure. Conventional layers are not packed as homogeneously, perhaps because of the greater difficulty of preparing layers from sorbents with a broader particle size distribution. The C coefficient is a measure of the resistance to mass transfer between the stationary phase and the streaming portion of the mobile phase. Typical values of C for columns are much smaller than for layers. The large resistance to mass transfer term might arise as a result of restricted diffusion within the porous particles or from partial blocking of the intraparticle channels, owing to the presence of binder. This is probably of little account for capillary flow separations owing to the low prevailing mobile phase velocities but is significant for separations by forced flow. The B coefficient is a measure of the contribution of longitudinal diffusion to the plate height and should be similar for layers and columns, as observed.

6.3.5 Zone Broadening in Multiple Development

Unidimensional multiple development provides a complementary approach to forced flow for minimizing zone broadening [61,67-70]. All multiple development techniques employ successive repeated development of the layer in the same direction with the removal of mobile phase between developments. Approaches differ in the changes made, e.g. mobile phase composition and solvent-front migration distance, between consecutive development steps; the total number of consecutive development employed can also be varied. Capillary forces are responsible for migration of the mobile phase but a zone-focusing mechanism is used to counteract the normal zone broadening that occurs in each successive development. Each time the solvent front traverses the stationary sample zone, the zone is compressed in the direction of development. The compression occurs because the mobile phase contacts the bottom edge of the zone first; here the sample molecules start to move forward before those molecules still ahead of the solvent front. When the solvent front has reached beyond the zone, the focused zone migrates and is subject to the normal zone broadening mechanisms. Both theory and experiment indicate that beyond a minimum number of development steps zone widths converge to a constant value that is roughly independent of migration distance, Figure 6.5 [71]. The position of a zone in multiple chromatography (constant development length) is given by

$$R_{F,P} = 1 - (1 - R_F)^P \quad (6.7)$$

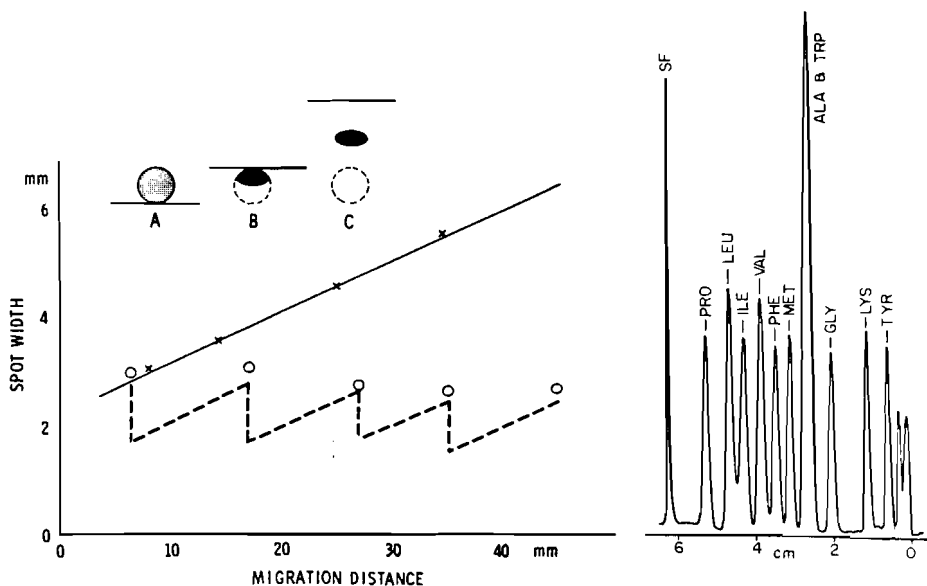


Figure 6.5. Illustration of the zone refocusing mechanism in multiple development (left) and its application to the separation of a mixture of phenylthiohydantoin-amino acid derivatives (right). The broken line represents the change in zone size due to the expansion and contraction stages in multiple development and the solid line depicts the expected zone width for a zone migrating the same distance in a single development. (From ref. [71]; ©Research Institute for Medicinal Plants).

and in incremental multiple development (each successive development distance is increased by a constant amount) by

$$R_{F,p} = 1 - (1 - R_F) \{ 1 - (1 - R_F)^p \} / p R_F \quad (6.8)$$

where $R_{F,p}$ is the apparent R_F value after p successive developments and R_F is the R_F value in the first development. Equations used to predict zone widths are more complicated and better handled by computer [69,70]. The theory developed so far is for isocratic mobile phases and is not applicable to solvent gradients, although the same general phenomenon of a roughly constant zone width is observed in optimized separations by automated multiple development. For difficult separations by capillary flow, multiple development is the general strategy used to increase the zone capacity.

6.3.6 Resolution and Zone Capacity

The resolution, R_S , between two separated zones in thin-layer chromatography is defined as follows

$$R_S = 2(Z_{X2} - Z_{X1}) / (w_{b1} + w_{b2}) \quad (6.9)$$

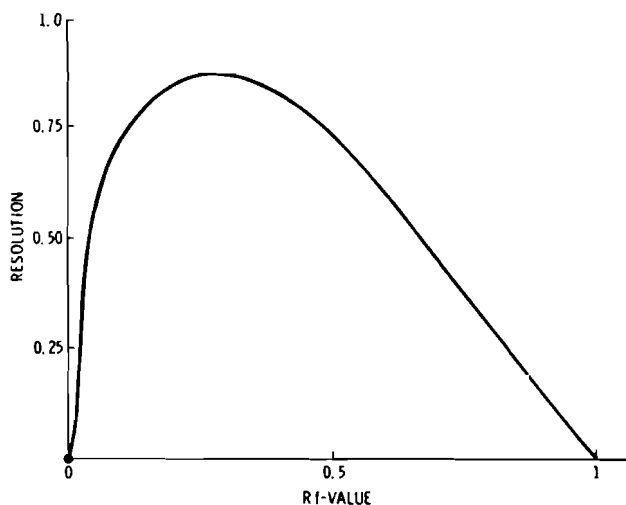


Figure 6.6. Variation of the resolution of two closely migrating zones as a function of the R_F value of the faster moving zone.

where Z_X is the zone center migration distance, w_b the width of the zone at its base, and the subscripts 1 and 2 refer to the individual zones with the larger number corresponding to the zone with the higher R_F value [72]. To optimize resolution, it is necessary to relate resolution to the experimental parameters, such as the layer efficiency, the ratio of the equilibrium constants for the separation process, and the position of the zones in the chromatogram. The thin-layer analog of classic resolution equation for column chromatography is given by

$$R_S = (\sqrt{N} / 4) (R_{F2} - R_{F1}) / R_{F2} \quad (6.10)$$

However, in thin-layer chromatography neither N nor the retardation factors are independent of the ratio of the equilibrium constants for the separation process or the properties of the layer. The value of N is strongly dependent on R_F for capillary flow. As a crude approximation N can be replaced by $N_1 R_{F2}$ where N_1 is the plate number the zones would have passed over if they migrated to the solvent front position. Substituting for N in equation (6.10) and rearranging gives

$$R_S = [\sqrt{(N_1 R_{F2})} / 4] [(k_1 / k_2) - 1] [1 - R_{F2}] \quad (6.11)$$

where k is the retention factor. Equation (6.11) is adequate for a qualitative description of the effect of layer quality, selectivity, and zone position on resolution for a single development with capillary flow, Figure 6.6. Resolution increases with efficiency in a manner that depends linearly on the R_F value. Relatively small changes in selectivity have a large impact on the ease of obtaining a separation, since the total plate number

available is not very large. Separations by thin-layer chromatography are easy when $(R_{F2} - R_{F1}) > 0.1$, and very difficult or impossible for $(R_{F2} - R_{F1}) < 0.05$, in the region of the optimum R_F value for the separation. The effect of zone location on resolution shows the opposite behavior to layer quality. At large values of R_{F2} , the term $(1 - R_{F2})$ is small and resolution falls to zero at $R_{F2} = 1$. Differentiation of Eq. (6.11) indicates that the maximum resolution of two difficult to separate zones will occur at a R_F value of about 0.3. From Figure 6.6, it can be seen that resolution does not change significantly for R_F values between 0.2 and 0.5; and within this range, the resolution is greater than 92% of the maximum value (75% between $R_F = 0.1$ and 0.6).

Resolution in forced flow thin-layer chromatography is not restricted by the same factors that apply to capillary flow. Resolution increases almost linearly with the solvent-front migration distance and is highest for separations at the optimum mobile phase velocity. Resolution has no theoretical limit for forced flow; the upper bounds are established by practical constraints (plate length, separation time and inlet pressure).

The potential of a chromatographic system to provide a separation can be estimated from its zone capacity, also referred to as the separation number (SN) or spot capacity in thin-layer chromatography. The separation number envisages the chromatogram as being similar to a string of beads, each bead touching its neighbor, with no unoccupied space between the beads. In practice, it leads to an inflated estimate of the real separation capacity, since real chromatograms do not consist of equally spaced peaks. It does provide, however, a plausible guide for comparison of different thin-layer separation systems. In addition, it provides an indication of the possibility of separating a given mixture, for unless the separation number exceeds the number of sample components by a significant amount, the separation will be difficult to impossible to achieve.

The separation number is defined as the number of spots completely separated with $R_S = 1$ between $R_F = 0$ and $R_F = 1$. Most methods of calculation, however, make approximations for either the form of the chromatogram, such as a geometric increase in zone size throughout the chromatogram [7,32,73], or attempt to estimate the increase in zone size by a theoretical model [74,75]. No approach is completely satisfactory, and only the simplest case is considered here. The separation number is calculated from Eq. (6.12), or more simply Eq. (6.13), with b_0 and b_1 as defined in Figure 6.2.

$$SN = \log (b_0 / b_1) / [\log (1 - b_1 + b_0) / (1 + b_1 - b_0)] \quad (6.12)$$

$$SN = [(Z_f - Z_o) / (b_0 + b_1)] - 1 \quad (6.13)$$

Equation (6.13) provides values that are about 25% smaller than those calculated by Eq. (6.12). Some typical results from theory or determined by experiment are summarized in Table 6.4. Results from theory are probably too high and represent an upper limit. Experiment indicates a zone capacity of about 12–14 for a single development with capillary flow. This rises to about 30–40 for forced flow. Automated multiple development with capillary flow provides a similar zone capacity to forced flow. Two-dimensional thin-layer chromatography employing different retention

Table 6.4

Experimental or theoretical zone capacity for different conditions in thin-layer chromatography

Separation mode	Dimension	Zone capacity
<i>(i) Predictions from theory</i>		
Capillary flow	1	< 25
Forced flow	1	< 80 (up to 150 depending on pressure limit)
Capillary flow	2	< 400
Forced flow	2	Several thousand
<i>(ii) Experimental observations</i>		
Capillary flow	1	12 - 14
Forced flow	1	30 - 40
Capillary flow (AMD)	1	30 - 40
Capillary flow	2	≈ 100
<i>(iii) Predictions based on (ii)</i>		
Forced flow	2	≈ 1500
Capillary flow (AMD)	2	≈ 1500

mechanisms for each orthogonal separation provides a significantly larger zone capacity than any of the unidimensional development methods. This accounts for the continuing interest in this technique (see section 6.6.4). In theory, the zone capacity becomes very large for two-dimensional thin-layer chromatography if forced flow or multiple development is used for the orthogonal separations, but this has not been demonstrated by experiment [1].

6.4 STATIONARY PHASES

Thin-layer plates can be prepared in the laboratory by standardized procedures [5,6], although the exacting experimental conditions required for the preparation of layers with reproducible separation properties are more easily obtained in a manufacturing setting. Today, most laboratories use commercially available precoated plates. Precoated plates for high performance, conventional and preparative thin-layer chromatography are available in thicknesses from 0.1 to 2.0 mm on either glass, aluminum or plastic backing sheets [76]. Most plates also contain a binder such as poly(acrylic acid) and its salts, gypsum or starch in amounts from 0.1 to 10% (w/w), to impart the desired mechanical strength, durability and abrasion resistance to the layer. A UV-indicator, such as manganese-activated zinc silicate of a similar particle size to the sorbent, may be added to the layer for visual evaluation of sample zones by fluorescence quenching. Thin-layer plates with a binary layer of two different, separated sorbents, forming a narrow interface parallel to one edge, are available for two-dimensional separations. If one of the layers is a type of silica with very weak retention properties, it can be used as a concentrating zone, to aid sample application [77]. Recent commercial

Table 6.5

Typical properties of inorganic oxide adsorbents used for thin-layer chromatography

Property	Silica gel	Alumina	Kieselguhr
Specific surface area (m ² / g)	200-800	50-350	1-4
Specific pore volume (ml / g)	0.5-2	0.1-0.4	1-3
Average pore diameter (nm)	4-20	2-35	10 ³ -10 ⁴
Concentration of active sites (μmol / m ²)	8	13	

Silica gel high performance layers

	Merck Si 60	Merck Si 50000	Whatman HP-K
Specific surface area (m ² / g)	550	0.5	300
Specific pore volume (ml / g)	0.82	≈0.63	0.70
Average pore diameter (nm)	6	5000	8
Average particle size (μm)	5	5	5
Layer thickness (mm)	0.2	0.2	0.2
Apparent pH (10 % aqueous suspension)	7.0		7.0-7.2

developments include high performance layers prepared from spherical silica particles and mixed silica and magnesium tungstate layers for improved sensitivity with wider spectral windows for *in situ* diffuse reflectance FTIR measurements [78]. Binder-free, ultra-thin monolithic silica layers for fast separations prepared by sol-gel technology have been prepared [79]. Glass rods with a sintered adsorbent coating are used for thin-layer chromatography, primarily in conjunction with a scanning flame ionization detector (section 6.9.5) [80,81].

6.4.1 Inorganic Oxide Layers

Inorganic oxide adsorbents commonly used in thin-layer chromatography include silica gel, alumina, kieselguhr, and Florisil [9,82]. Of these, silica gel is by far the most important. The chromatographic properties of inorganic oxides depends on their surface chemistry and composition, specific surface area, specific pore volume, and average pore diameter, Table 6.5. Retention on silica gel depends on the number, type and spatial location of analyte functional groups. Surface silanol groups are the dominant sites for analyte interactions. Since these are present at roughly the same concentration for all silica surfaces, general retention largely reflects differences in the specific surface area. More specific differences in the type and distribution of silanol groups are responsible for selectivity differences [83]. Consequently, it is not always possible to reproduce the same separation on different silica gel layers. The influence of analyte functional group type and spatial position on retention is illustrated in Figure 6.7 for the separation of ethynyl steroids, which are the active ingredients in oral contraceptives [1,84]. The steroids with phenolic groups are the most strongly retained, followed by hydroxyl groups, and ketone and ester groups. Subtle separation differences are also seen due to steric hindrance at a functional group and differences in ring conformations. Silica gels of extremely low surface area (e.g. Si 50000) are used for the separation of polar

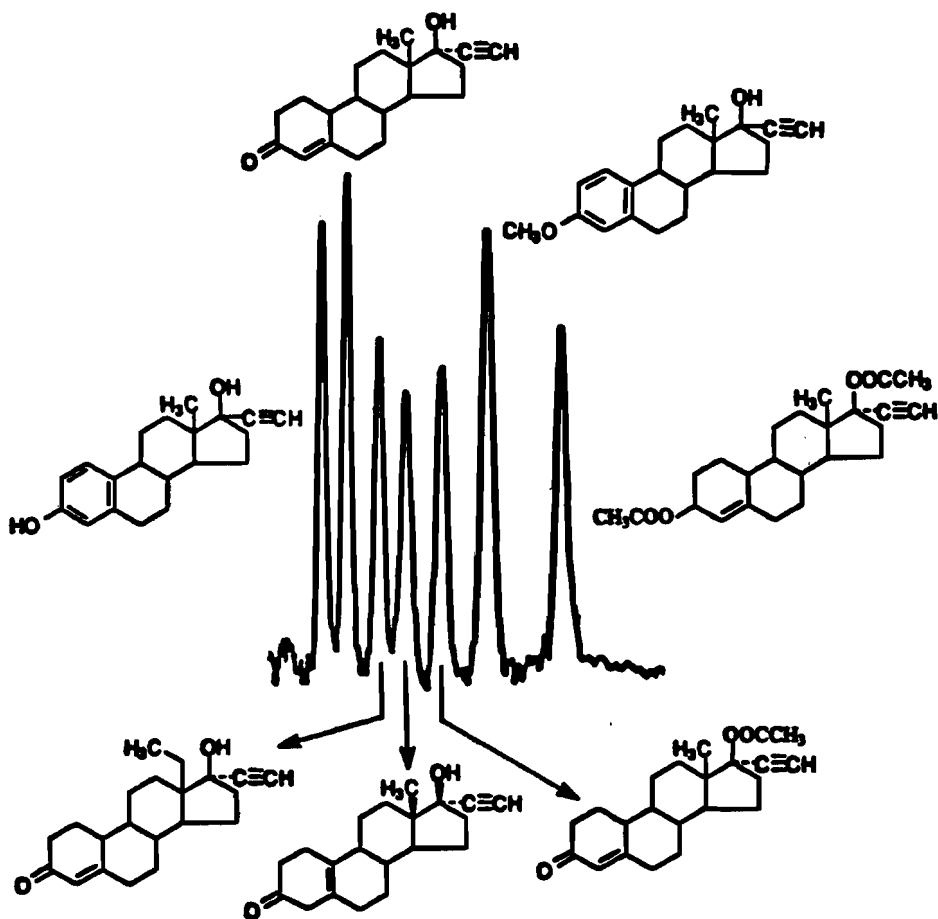


Figure 6.7. Separation of ethynyl steroids on a silica gel 60 HPTLC plate using two 15-minute developments in hexane-chloroform-carbon tetrachloride-ethanol (7:18:22:1) as mobile phase. (From ref. [1]; ©Elsevier).

compounds (e.g. carbohydrates, nucleic acid derivatives, phosphates and sulfonates, etc.) that are not easily separated on high-activity silica gel layers [85]. This type of silica gel is the same as that used for the preparation of concentrating zones on bilayer plates. With the exception of Si 50000 the range of adsorbents with different pore sizes is not great. Most materials have an average pore size of 6 nm designed for the separation of small molecules (MW < 750). The thickness of high performance layers is typically 0.2 mm. Reducing the layer thickness to 0.1 mm increases the separation speed (mobile phase velocity constant increases about 1.1-2.5 fold) but has little influence on the zone capacity [86]. The thinner layers afford an increase in detection limits by scanning densitometry of about 1.1 to 1.5 fold. These small gains in speed and limits of detection

are offset by a loss in ease of use. Impregnating silica gel layers with a solution of a complexing agent is a convenient method for modifying the selectivity of the layer for specific applications [5,6,87-89]. A few common examples include silver nitrate for the separation of saturated and unsaturated compounds, boric acid to differentiate between isomers with vicinal hydrogen-bonding functional groups, and caffeine or picric acids for the separation of polycyclic aromatic compounds.

The separation properties of alumina are complementary to those of silica gel [82,90,91]. The alumina surface is more complex containing hydroxyl groups, aluminum cations, and oxide anions. Its apparent pH and hydration level significantly influences the separation properties of alumina. Alumina is available with various adjusted pH ranges, corresponding to basic (pH \approx 9-10), neutral (pH \approx 7-8) and acidic (pH \approx 4-4.5). The separation mechanism for alumina is not as well understood as silica gel. Kieselguhr (a naturally occurring impure form of silica gel) and Florisil are not widely used in contemporary practice [82,91].

6.4.2 Chemically Bonded Layers

Chemically bonded layers are prepared by reacting silanol groups on the silica gel surface with various organosilane reagents [9,82,92-94]. Silica-based, chemically bonded layers with dimethyl-, diphenyl-, ethyl-, octyl-, octadecyl-, 3-aminopropyl-, and 3-cyanopropylsiloxane-bonded groups and a siloxane-bonded spacer bonded propanediol group are commercially available. The selection of silica gel substrate and method of preparation result in nominally similar generic products with different separation properties, Table 6.6. Reversed-phase layers from Merck are prepared from dichloroalkylmethylsilanes with methyl- (RP-2), octyl- (RP-8), and octadecyl- (RP-18) alkyl groups. The organosilane reagent is bonded to the surface by a combination of one or two bonds. The 3-aminopropylsiloxane-bonded layer is prepared with 3-aminopropyltriethoxysilane and has a much higher surface coverage than the 3-cyanopropylsiloxane-bonded layer prepared from 3-cyanopropylchloromethylsilane. The 3-cyanopropylsiloxane group is bound almost entirely to the silica substrate by bidentate linkages. On the other hand, the 3-aminopropylsiloxane group is bound to the silica substrate by a combination of bidentate and tridentate linkages. In the latter case, this is most likely the result of crosslinking of the reagent during the surface reaction. The two Macherey-Nagel octadecylsiloxane-bonded layers are of the polymeric type, prepared from a trifunctional reagent. They differ primarily in the extent of surface bonding. The Whatman products are prepared by different chemistries. The dimethylethylsiloxane-bonded and dimethyloctylsiloxane-bonded layers are prepared from monofunctional organosilane reagents. The octadecylsiloxane-bonded layer is prepared from a trifunctional organosilane reagent and has a crosslinked, polymeric structure. In addition, the octylsiloxane-, octadecylsiloxane- and diphenylsiloxane-bonded layers are endcapped, but even so, they possess a relatively high concentration of silanol groups.

Reversed-phase alkylsiloxane-bonded layers with a high level of surface bonding are incompatible with mobile phases containing a significant amount of water. In this

Table 6.6

Characteristic properties of precoated chemically bonded layers

Manufacturer	Derivatizing reagent	Percent silanol groups reacted	Carbon loading (%)	Average particle size (μm)
Merck				
RP-2	Bifunctional	50		5-7
RP-8 W	Bifunctional	25	8.9	11-13
RP-8	Bifunctional	37		5-7
RP-18 W	Bifunctional	22	15.4	11-13
RP-18	Bifunctional	35		5-7
3-Aminopropyl	Trifunctional	50	5.8	5-7
3-Cyanopropyl	Bifunctional	27		5-7
Whatman				
KC-2	Monofunctional		4.5	10-14, or 20
KC-8	Monofunctional		8.5	10-14
KC-18	Trifunctional	16	12.5	10-14
Diphenyl	Difunctional		8.5	10-14
Macherey-Nagel				
Sil C18-100	Trifunctional	45		5-10
Sil C18-50	Trifunctional	30		5-10

case, the hydrophobic repulsive forces are stronger than the capillary forces moving the solvent through the layer (this does not apply to forced flow separations). For mobile phases containing more than about 40% (v/v) water, the mobile phase velocity is very slow and/or the precoated layers swell and flake off from the support. This problem is overcome by using a slightly larger particle size for the silica substrate, a reproducible, although lower level of silanization than the maximum value, and a different binder to enhance its water stability. These layers are referred to as water wettable and are used for all types of reversed-phase separations, while layers with a high level of silanization are used for normal-phase separations with predominantly non-aqueous mobile phases. For reversed-phase separations, the range of mobile phase velocities depends in a complex manner on the mobile phase composition, Figure 6.8 [93-95]. Contributing to the shape of the curves are properties of the binder as well as the mobile phase (e.g. surface tension/viscosity ratio and possibly the contact angle). Polar chemically bonded phases are compatible with water in all proportions and are suitable for use in both normal- and reversed-phase chromatography. In addition, the mobile phase velocity varies less with composition. Hydrolysis of bonded phases by partially aqueous, extreme pH, mobile phases was shown to affect separations using multiple development [96].

Chemically bonded layers are used essentially for separations that cannot be performed on silica gel, Table 6.7. Compounds of extreme polarity and ionic compounds are difficult to separate on silica gel because of strong retention and limited selectivity. Chemically bonded phases provide complementary properties to silica gel for the separation of compounds of intermediate polarity. In addition, 3-aminopropylsiloxane-bonded layers are used as weak ion-exchangers for the separation of anions with acid-

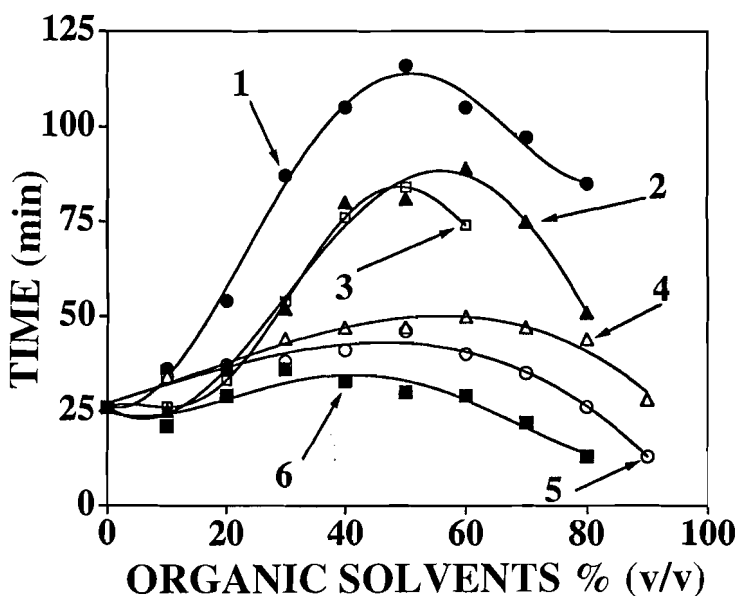


Figure 6.8. Plot of the time required for the solvent front to migrate 5 cm as a function of mobile phase composition for 2-propanol (1), N,N-dimethylformamide (2), 2,2,2-trifluoroethanol (3), methanol (4), acetone (5) and acetonitrile (6) in water on a Merck HPTLC RP-18 WF254s layer. (From ref. [95]; ©Research Institute for Medicinal Plants).

buffered mobile phases [12,97,98]. A useful property of 3-aminopropylsiloxane-bonded layers is the ease of converting different substances that may require derivatization for detection (e.g. carbohydrates, amino acids, and catecholamines) into fluorescent derivatives simply by heating while adsorbed on the layer [99].

6.4.3 Other Materials

Native and microcrystalline cellulose precoated plates are used in the life sciences for the separation of polar compounds (e.g. carbohydrates, carboxylic acids, amino acids, nucleic acid derivatives, phosphates, etc) [85]. These layers are unsuitable for the separation of compounds of low water solubility unless first modified, for example, by acetylation. Several chemically bonded layers have been described for the separation of enantiomers (section 10.5.3). Polyamide and polymeric ion-exchange resins are available in a low performance grade only for the preparation of laboratory-made layers [82]. Polyamide layers are useful for the reversed-phase separation and qualitative analysis of phenols, amino acid derivatives, heterocyclic nitrogen compounds, and carboxylic and sulfonic acids. Ion-exchange layers prepared from poly(ethyleneimine), functionalized poly(styrene-divinylbenzene) and diethylaminoethyl cellulose resins and powders and are used primarily for the separation of inorganic ions and biopolymers.

Table 6.7

Retention properties of silica-based chemically bonded layers

Type of modification	Functional group	Application
Alkylsiloxane	Si-CH ₃	• For reversed phase separations generally but not exclusively
	Si-C ₂ H ₅	• Separation of water soluble polar organic compounds (RPC)
	Si-C ₈ H ₁₇	• Weak acids and bases after ion suppression (RPC)
	Si-C ₁₈ H ₃₇	• Strong acids and bases by ion-pair mechanism (RPC) • Homologs and Oligomers (RPC) • Hydrocarbon-like and polycyclic aromatic compounds (RPC & NPC)
Phenylsiloxane	Si-C ₆ H ₅	• No specific applications that cannot also be preformed on alkylsiloxane-bonded layers
Cyanopropylsiloxane	Si-(CH ₂) ₃ CN	• Useful for both RPC and NPC • In NPC it exhibits properties similar to a low capacity silica gel. • In RPC it exhibits properties similar to short-chain alkylsiloxane-bonded layers (it has no selectivity for dipole-type interactions)
Aminopropylsiloxane	Si-(CH ₂) ₃ NH ₂	• Used mainly in NPC & IEC. Limited retention in RPC • Selectively retains compounds by hydrogen-bond interactions in NPC. Separations unlike those obtained on silica gel. • Functions as a weak anion exchanger in acidic mobile phases (IEC)
Spacer bonded propanediol	Si-(CH ₂) ₃ OCH ₂ CH(OH)CH ₂ OH	• Used in NPC and RPC but more useful for NPC because of low retention in RPC. • Reasonable retention of polar compounds by hydrogen bond and dipole-type interactions in NPC. More hydrogen-bond acidic and less hydrogen-bond basic than aminopropylsiloxane-bonded layers in NPC. More retentive than aminopropylsiloxane-bonded layers in RPC. • Similar retention to short chain alkylsiloxane-bonded layers but different selectivity for hydrogen-bonding compounds

Several types of crosslinked gels [e.g. dextrans (Sephadex), poly(acrylamide) (Bio-Gel P), and poly(acryloylmorpholine)(Enzacryl)] are used for the separation of biopolymers by thin-layer chromatography. These materials must be swollen in an appropriate buffer/solvent system before spreading as a layer. Laboratory-made layers require modified equipment for their use and afford only modest performance characteristics.

6.4.4 Layer Pretreatments

Before chromatography, it is common practice to prepare the layers for use by any or all of the following pretreatments: washing, activation, conditioning, and equilibration. In addition, layers may be cut to appropriate sizes using scissors for plastic- or aluminum-backed plates and diamond or carbide glass cutting tools for glass-backed layers [100]. Precoated plates are invariably contaminated, or quickly become so, because of residual contaminants from the manufacturing process, contact with packaging materials, and

adsorption of materials from the atmosphere. Contamination may result in irregular and drifting densitometric baselines, ghost peaks in the chromatogram (recognized from a chromatogram obtained by scanning the layer between sample tracks), and reduced sensitivity for postchromatographic derivatization [101]. These problems are easily remedied by predevelopment and/or immersion of the layer in a polar solvent such as methanol or 2-propanol [100-102]. Predevelopment with a strong solvent or the intended mobile phase for the separation, often results in a pile up of contaminants in a region close to the top of the plate. This can result in an upwardly sloping baseline in scanning densitometry. A single or double immersion in a polar solvent for about 5 minutes is generally superior to predevelopment for removing contaminants. For trace analysis, both sequential immersion and predevelopment may be required for acceptable results [102,103].

The absolute R_F value and its reproducibility on inorganic oxide layers depends on the layer activity. This is controlled by the adsorption of reagents, most notably water, through the gas phase [100]. Physically adsorbed water is removed from silica gel layers by heating at about 120°C for 30 minutes. Afterwards the plates are stored over a drying agent in a grease-free desiccator. Heat activation is not usually required for chemically bonded layers. Deactivation of silica gel layers by exposure to the atmosphere is extremely rapid. In modern environment-controlled laboratories, layers achieve a consistent level of activity almost instantaneously, that should provide sufficient reproducibility for most separations. Indeed, brief exposure of activated layers to the laboratory atmosphere may render the activation process a waste of effort.

Inorganic oxide layers can be adjusted to a defined activity level by exposure to a defined gas phase in an enclosed chamber. This is best performed after sample application in a developing chamber that allows both conditioning and development of the layer in the same chamber (e.g. a twin trough chamber). Alternatively, separate conditioning and development chambers can be used. Atmospheres of different constant relative humidity can be obtained by exposure to the vapor phase in equilibrium with solutions of concentrated sulfuric acid or saturated solutions of various salts [100]. In the same way, acid or base deactivation is carried out by exposure to concentrated ammonia or hydrochloric acid fumes.

6.5 SAMPLE APPLICATION

Samples are applied to thin-layer plates as spots or bands to conform to the demands of minimum size and a homogeneous distribution of sample within the starting zone [8,9,104,105]. For high performance layers, starting spot sizes of 1 – 2 mm are desirable, corresponding to a sample volume of 100 to 200 nl if applied by a dosimeter. For conventional layers, sample volumes 5 to 10-fold higher are acceptable. Favorable properties for sample solvents are summarized in Table 6.8. Sample application by hand-held devices is unsuitable for use with scanning densitometry, which requires that the starting position of each spot be accurately known. This is easily achieved with

Table 6.8

Solution requirements for sample application

Property	Requirements
Sample solvent	<ul style="list-style-type: none"> • Good solvent for the sample to promote quantitative transfer from the sample application device to the layer • Low viscosity and sufficiently volatile to be easily evaporated from the layer • Wet the sorbent layer to provide adequate penetration of the layer by the sample (a potential problem for alkylsiloxane-bonded layers and aqueous sample solutions) • Weak chromatographic solvent to minimize predevelopment during sample application (ideally if used as a chromatographic solvent the least retained sample component should have an R_F value < 0.1)
Aqueous solutions	<ul style="list-style-type: none"> • Dilute if possible with a water-miscible solvent that forms a lower boiling azeotrope • Apply in small increments or if spray-on techniques are used with a slow application rate • Use layers with a concentrating zone and refocus the sample prior to development
Viscous solutions	<ul style="list-style-type: none"> • Dilute if possible with a volatile solvent of low viscosity • Use a direct transfer technique (e.g. contact spotter)
Suspensions	<ul style="list-style-type: none"> • Filter before attempting sample application • Otherwise use layers with a concentrating zone and an extraction solvent that mobilizes the components of interest for refocusing

mechanical devices operating to a precise grid mechanism. In addition, the sample must be applied to the layer without damaging the surface, something that is near impossible to achieve with manual sample application devices. When using dosimeters, it is important that samples and standards are applied to the layer in identical volumes and in the same solvent. This ensures identical starting zones are formed. Calibration curves should not be prepared by applying variable volumes from a single standard solution, since this is likely to result in poor linearity and non-zero intercepts. This restriction does not apply to spray-on devices.

6.5.1 Application Devices

Sample application devices for thin-layer chromatography encompass a wide range of sophistication and automation. The most popular devices for quantitative thin-layer chromatography use the spray-on technique. A nitrogen atomizer sprays the sample from a syringe or capillary onto the layer surface as narrow, homogeneous bands. Linear or zigzag motion of either the plate on a motorized stage or the atomizer head allows bands of any length between 0 (spots) and the maximum transit length of the spray head (or plate). Typical band lengths are 0.5 or 1.0 cm with longer bands used mainly for preparative-scale separations. The rate of sample deposition is also adjustable to accommodate sample solutions of different volatility and viscosity. Band application allows the sample band to be made longer than the slit length of the light source, minimizing quantification errors due to positioning of the sample within the light beam in scanning densitometry. Also, different volumes of a single standard

can be applied for calibration and the standard addition method of quantification is facilitated by overspraying the sample already applied to the layer with a solution of the standard.

Several fully automated spray-on sample applicators are available. In one device, a motor driven syringe is used to suck up sample volumes of 0.1 to 50 μl , which are then deposited as spots or bands on the layer [104]. The syringe feeds a stainless-steel capillary connected to a capillary atomizer. The applicator can be programmed to select samples from a rack of vials and deposit fixed volumes of the sample, at a controlled rate, to selected positions on the layer. The applicator automatically rinses itself between applications and can spot or band a whole plate with different samples and standards without operator intervention. A number of multi-sample applicators for the simultaneous transfer and deposition of several samples at the same time have been described [106-108].

Glass microcapillaries and fixed-volume dosimeters are also used for sample application. These require less sophisticated instruments. Fixed-volume dosimeters with 100 or 200 nl volumes for use with high performance layers are fabricated from platinum-iridium capillaries sealed into a glass support capillary of larger bore [7]. The metal capillary tip is polished to provide a smooth flat surface of small area (ca. 0.05 mm²), which is brought into contact with the layer by a mechanical device to discharge its volume. Mechanical application of the sample is possible by attaching a metal collar to the glass support capillary. This allows the dosimeter to be held by a magnet and lowered to the plate surface under controlled conditions. A spring mechanism allows the applicator head to be lowered and lifted from the plate surface while the frictional force holding the dosimeter to the applicator head controls the force with which the dosimeter engages the layer. A click-stop grid mechanism allows the even spacing of sample spots on the layer, and provides a frame of reference for sample location during scanning densitometry.

Layers with a concentrating zone simplify some aspects of sample application [77]. Microliter volumes can be applied either as spots or bands to any position on the concentrating zone. Alternatively, the entire zone can be immersed in a dilute solution of the sample. During development, the sample migrates out of the concentrating zone and is focused to a narrow band at the interface between the concentrating zone and separation layer. However, since the distribution of the sample within the band may be uneven, quantitative measurements by scanning densitometry are often poor. Thin-layer plates with concentrating zones are mainly used for qualitative applications, for the application of large sample volumes, and for the application of crude samples (e.g. biological fluids).

6.6 MULTIMODAL (COUPLED COLUMN-LAYER) SYSTEMS

The specific reasons for coupling column separations with thin-layer chromatography are to increase peak capacity and to take advantage of layer attributes summarized

in Table 6.1 (section 6.2). The thin-layer plate functions as a separation and storage device retaining information from the column and thin-layer separations in an immobilized format. This allows sample components to be investigated free of time constraints, which is advantageous for: biomonitoring (section 6.9.6); for samples that require derivatization for detection; for sequential evaluation using different detection principles; to preserve and transport the separation to different locations for evaluation; and for applications employing solid-phase spectroscopic identification techniques (section 9.3.1.4).

Coupling gas chromatography to thin-layer chromatography (GC-TLC) is straightforward but has not been widely used since the late 1960s when several interfaces were described [61]. Many of the problems for which these instruments were used are solved by gas chromatography-mass spectrometry today, and contemporary interest in GC-TLC has declined. In the late 1970s an apparatus for supercritical fluid extraction with deposition of the fluid extract onto a moving thin-layer plate was described [109,110]. A suitable interface for direct coupling of supercritical fluid chromatography to thin-layer chromatography (SFC-TLC) was described more recently [111,112]. Decompression of the supercritical fluid at a capillary orifice occurs with rapid cooling, favoring the deposition process without disturbing the conversion of the fluid to a gas. Efficient transfer of sample to the layer requires solvent addition, to minimize the loss of high-speed sample particles. Wet particle deposition being much more efficient than dry particle deposition. The coupling of capillary electrophoresis to thin-layer chromatography (CE-TLC) has been described, but no real applications developed [113]. Current interest is largely in the coupling of column liquid chromatography to thin-layer chromatography (LC-TLC), which has reached a reasonable level of maturity and commercialization.

6.6.1 Liquid Chromatography-Thin-Layer Chromatography (LC-TLC)

The most general interface for coupling column liquid chromatography to thin-layer chromatography (LC-TLC) is based on different modifications to the spray-jet applicator (section 6.5.1) [61,114-120]. At flow rates typical for packed capillary columns (5–100 $\mu\text{l}/\text{min}$) the total mobile phase can be applied to the layer. A splitter in the transfer line to the spray-jet applicator is required to accommodate higher flow rates from wider bore columns. The column eluent is nebulized by mixing with (heated) nitrogen gas and sprayed as an aerosol onto the layer. The spray head is moved horizontally on one line within a defined bandwidth or, better, is made to deposit the spray over a defined rectangular area (e.g. 8 x 6 mm) to promote effective solvent evaporation. In the latter case, the zone is focused by a short development with a strong solvent before separation.

In the profiling mode, the whole column chromatogram is divided into volume fractions sequentially transferred to the layer and deposited as a series of bands that are subsequently developed in parallel. Each track (band) is scanned individually revealing an immense amount of information about the sample composition. In the target compound mode, fractions identified by the column detector, or from elution

windows established by marker compounds, are transferred to the layer and stored there. When all the available space is occupied by column fractions from a single or several samples, the layer is developed and evaluated. The main limitations of the spray-jet interface are its restricted flow capability and inability to handle involatile ionic additives, such as buffers and ion-pair reagents. These problems can be overcome by combining an automated solid-phase extraction module with the spray-jet applicator to concentrate column fractions and exchange the solvent for layer deposition [121].

6.7 DEVELOPMENT TECHNIQUES

Development in thin-layer chromatography is the process by which the mobile phase moves through the layer, thereby inducing differential migration of the sample components. The principal techniques are linear, circular and anticircular with the mobile phase velocity controlled by capillary forces or external pressure. The application of any of these techniques can be extended using continuous or multiple development.

6.7.1 Linear and Radial Development

For linear development, samples are applied along one edge of the plate and separated in the direction of the opposite edge. Viewed in the direction of development, the chromatogram consists of a series of compact symmetrical spots of increasing diameter, or if samples are applied as bands, as rectangular zones of increasing width.

If the position of sample application and mobile phase entry point are at the center of the layer with the flow of mobile phase towards the periphery, then this mode of development is called circular chromatography [7]. Samples can be injected in the mobile phase, in which case they are separated as a series of concentric rings. Otherwise, samples are applied as a cluster of spots in a radial pattern around the solvent entry position. After development, spots near the origin remain symmetrical and compact while those near the solvent front are compressed in the direction of development and elongated at right angles to this direction [110].

For anticircular development, the sample is applied to the layer along the circumference of an outer circle and developed towards the center [7,123]. Spots near the origin remain compact while those close to the solvent front are elongated in the direction of migration, but of nearly constant width when viewed at right angles to the direction of development. Elongation of the sample spots at high R_F is unavoidable. It arises as a consequence of the lateral compression induced by flow of the mobile phase through a continuously decreasing layer area. The unique features of anticircular development are its high speed and high sample throughput. The mobile phase velocity remains approximately constant under capillary flow conditions because of the equivalence between the quadratic decrease in mobile phase velocity and a similar reduction in the layer area to be wetted.

Both circular and anticircular chromatograms require a scanning densitometer capable of radial or peripheral scanning, an option available with some instruments. Circular

development is used in rotational planar chromatography and is popular in preparative-scale chromatography because it allows separated samples to be continuously collected as they elute from the layer. Anticircular development is little used in contemporary practice and requires a specially designed developing chamber. Linear development is “the normal” and most widely used method.

6.7.2 Continuous Development

For continuous development, the mobile phase is allowed to traverse the layer under the influence of capillary forces until it reaches a predetermined position on the plate, where it is continuously evaporated. Evaporation of the mobile phase usually occurs at the plate atmospheric boundary by either natural or forced evaporation. The movement of the mobile phase to the air boundary occurs by capillary flow, but once it reaches the boundary, additional forces are applied by evaporation of the solvent. Eventually a steady state (constant velocity) is established, where the mass of solvent evaporating at the boundary is equivalent to the amount of new solvent entering the layer. Sandwich-type chambers for continuous development were reviewed by Soczewinski [124]. Perry [125] has outlined the use of the short-bed continuous development chamber for optimized continuous development with variable selection of the plate length, and Nurok [126] has proposed a theoretical model to optimize experimental conditions. Continuous development is used primarily to separate simple mixtures with a short development distance and a weaker (more selective solvent) than employed for conventional development [8]. It is not widely used in contemporary practice.

6.7.3 Multiple Development

For multiple development, the thin-layer plate is developed for a selected distance, then either the layer or the mobile phase is withdrawn from the developing chamber, and adsorbed solvent evaporated from the layer before repeating the development process [8,61,67,68,71,127]. It affords a versatile strategy for separating complex mixtures. The primary experimental variables of plate length, time for development (if continuous development is used), and mobile phase composition, can be changed at any development step, and the number of steps varied to obtain the desired separation. Quantitative measurements by scanning densitometry can be made at several steps in the sequence, if desired. Consequently, it is unnecessary for all components to be separated at one time, provided they can be identified (chromatographically or spectroscopically) at different segments of the development sequence. Multiple development provides higher resolution of complex mixtures than conventional or continuous development, can easily handle samples of a wide polarity range (stepwise gradient development), and because the separated zones are usually more compact, leads to lower detection limits. The higher zone capacity than is achieved by conventional development is the result of the zone focusing mechanism (see section 6.3.7) and the optimum use of solvent

Table 6.9
Multiple development techniques

Method	Features
Multiple chromatography	<ul style="list-style-type: none"> • Fixed development length • Same mobile phase for each development • The number of developments can be varied
Incremental multiple development	<ul style="list-style-type: none"> • Variable development length <ol style="list-style-type: none"> (a) First development is the shortest (b) Each subsequent development is increased by a fixed distance (c) Last development length corresponds to the maximum useful development distance • Same mobile phase for each development • The number of developments can be varied
Increasing solvent strength gradients	<ul style="list-style-type: none"> • Fractionates sample into manageable subsets • Optimizes separation of each subset • Complete separation of all components is not achieved at any segment in the development sequence
Decreasing solvent strength gradients	<ul style="list-style-type: none"> • Uses incremental multiple development • First development employs the strongest solvent with a weaker solvent for each subsequent step • Final separation recorded as a single chromatogram

selectivity. Equipment for automated multiple development is commercially available (section 6.7.5).

The main variants of multiple development are multiple chromatography and incremental multiple development, Table 6.9 [1]. Multiple chromatography is used for isocratic separations only while incremental multiple development is used for both isocratic and gradient separations. In multiple chromatography the plate is developed repeatedly over the same distance with the same mobile phase, the layer being separated from the mobile phase and dried between developments. Multiple chromatography can also be performed with a variable solvent entry position, by simultaneously incrementing the solvent entry position and solvent front position to maintain a fixed distance for each development [71,128,129]. The solvent entry position can be repositioned for each development by raising the solvent level for each development, by cutting off a portion of the bottom edge of the layer after each development, or by using a horizontal developing chamber with a layer repositioning device.

For incremental multiple development, the first development is the shortest and each subsequent development is incremented by (usually) a fixed distance or variable distances arrived at by trial-and-error, to obtain the desired separation. The last development is the longest and, in most cases, corresponds to the maximum useful development length for the layer.

Optimum conditions for isocratic multiple development are summarized in Table 6.10. The outcome of separations by multiple chromatography is the most predictable (see section 6.3.7) but is rarely the best approach for distributing sample zones throughout the whole chromatogram, and incremental multiple development is generally preferred. Incrementing the solvent entry position while simultaneously increasing

Table 6.10

Optimum separation conditions for isocratic multiple development

Mode	Features
Multiple chromatography	<ul style="list-style-type: none"> • Resolution is controlled primarily by the zone center separation since zone widths are relatively constant after the first few developments • The maximum zone center separation for two solutes of similar migration properties occurs when the zones have migrated 0.632 of the solvent front migration distance • The maximum observed resolution is largely independent of the average R_F value for the zones, although if the average R_F value is small a large number of developments will be needed to reach the maximum value • Compounds that are difficult to separate should be repeatedly developed with solvents that produce low R_F values corresponding to the most selective mobile phase for the separation
Incremental multiple development	<ul style="list-style-type: none"> • Increment the solvent entry position to maintain a fixed distance below the least retained zone: <ul style="list-style-type: none"> (a) Zone separation is increased owing to the longer migration distances achieved (b) Zone widths are reasonably constant throughout the chromatogram • Increment the solvent front migration distance: <ul style="list-style-type: none"> (a) Avoids moving portions of the chromatogram into the solvent front region (b) Resolution can be optimized throughout the chromatogram. The resolution reaches a plateau value and is not further degraded by increasing the number of developments (c) The number of developments can be selected based on the requirements of the most difficult zones to separate

the development distance in successive developments provides increased resolution of sample zones largely because of the increase in the zone migration distance. The normal zone broadening mechanism, leading to wider zones, is effectively counteracted by the focusing mechanism. Interestingly, resolution in incremental multiple development reaches a plateau value and is not subsequently reduced by increasing the number of development steps, unlike multiple chromatography.

Isocratic incremental multiple development with changes in the solvent entry position is selected for the separation of compounds with similar retention that tend to migrate as a compact group (e.g. isomers, diastereomers and analogs with minor structural variations). Separation is usually obtained only in the higher regions of the layer, and at each subsequent development, a significant amount of time is wasted while the advancing solvent front reaches the level of the lowest zone. As well as increasing the separation time, this process results in difficult separations being attempted in regions of the layer where the mobile phase velocity is inadequate to minimize zone broadening. An example of the separating power of different isocratic multiple development approaches is illustrated in Figure 6.9 [68]. The poorest separation is obtained by multiple chromatography. The separation is improved by using incremental multiple development, with the best separation (nearly to baseline) achieved by the simultaneous change of the solvent entry and solvent front migration distance at each successive development. This minimizes zone broadening and enhances the zone center separations by migrating the sample components over a longer plate length while

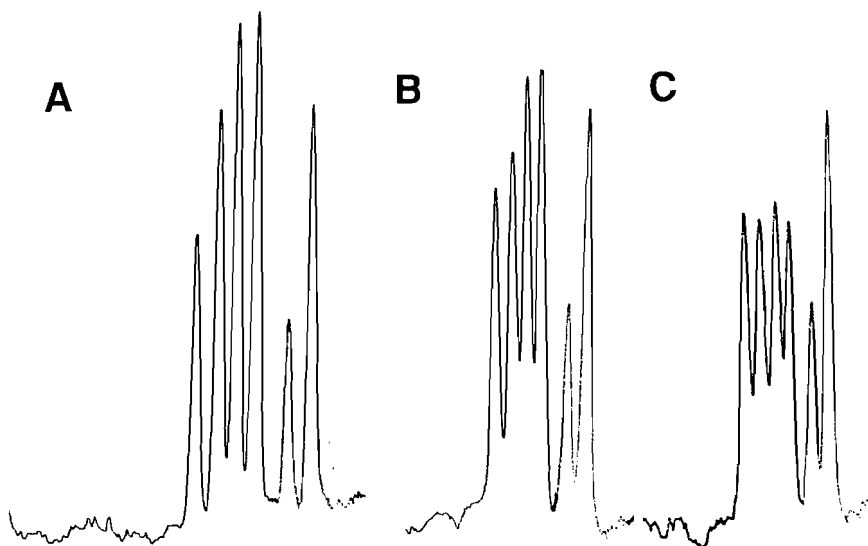


Figure 6.9. Separation of a mixture of estrogens by incremental multiple development with variable (A) and fixed (B) solvent entry position, and by multiple chromatography (C). A nine step sequence with the mobile phase cyclohexane-ethyl acetate (3:1, v/v) was used for (A) and (B) and seven 7 cm developments for (C), all on a silica gel HPTLC layer. The estrogens, in order of migration, are 17β -dihydroequilenin, 17α -dihydroequilenin, 17β -estradiol, 17α -estradiol, equilenin and estrone. (From ref. [68]. ©Research Institute for Medicinal Plants).

maintaining a mobile phase velocity that closely approaches the most favorable value for the separation.

For samples containing components of a wide retention range, some form of gradient development is required to obtain a separation of all components either in a single chromatogram or in separate chromatograms for successive developments. Figure 6.10 illustrates one such example [68,130]. The mobile phase can be optimized to separate either the low oligomer number or high oligomer number components in a single development, but not both simultaneously. Using multiple development with a stepwise mobile phase gradient, all of the oligomers are separated in a single chromatogram. Continuous composition gradients are rarely used in thin-layer chromatography, unlike column liquid chromatography. They require special equipment and less convenient experimental conditions than step gradients. In addition, step gradients can be constructed to mimic a continuous gradient with the added advantage that the zone focusing mechanism can be exploited to minimize zone broadening. For forced flow, which closely resembles column liquid chromatography, continuous composition gradients are commonly used and multiple development rarely.

Common methods for constructing solvent strength gradients in multiple development are summarized in Table 6.9. Gradients of increasing solvent strength are used to fractionate complex mixtures by separating just a few components in each

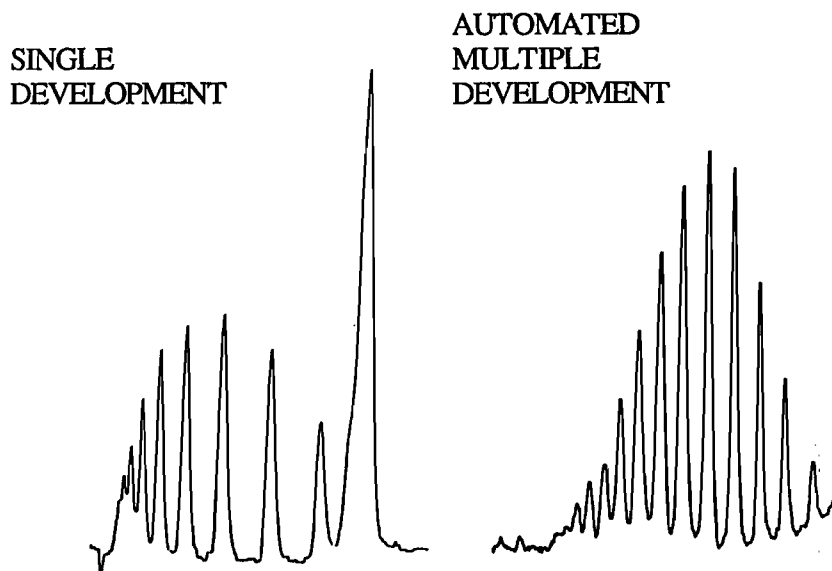


Figure 6.10. Comparison of normal development and incremental multiple development with a decreasing solvent strength gradient (AMD) for the separation of poly(ethylene glycol) 400 as its 3,5-dinitrobenzoate ester. The AMD separation employed a 15- step gradient with methanol, acetonitrile and dichloromethane as solvents. (From ref. [68]. ©Research Institute for Medicinal Plants).

step [8,71,131]. Individual compounds are usually identified and quantified by scanning intermediate steps at which the sample components of interest are separated. In this way the zone capacity can be much greater than predicted for a complete separation recorded as a single chromatogram. On the other hand, this approach can be tedious when many components are of interest, and it is difficult to automate. The alternative strategy is based on incremental multiple development with separation of the sample for the shortest distance in the strongest mobile phase with each subsequent, longer development using mobile phases of decreasing solvent strength. This strategy is most useful when it is desired to record the separation as a single chromatogram. On the other hand, the peak capacity is limited because all components must fit between the position of the sample origin and the final solvent front. The two approaches for exploiting solvent strength gradients are complementary, and a selection is made based on the properties of the sample. The decreasing solvent strength gradient approach is the operating basis of the automated multiple development chamber (section 6.7.5).

6.7.4 Two-Dimensional Development

For two-dimensional thin-layer chromatography the sample is spotted at the corner of the layer and developed along one edge of the plate [61,71,127,132,133]. The solvent is then evaporated, the plate rotated through 90°, and redeveloped in the orthogonal

Table 6.11

Methods for generating two different retention mechanisms in orthogonal directions

-
- Develop in orthogonal directions with two solvent systems exhibiting different selectivity for the sample components.
 - Use a bilayer plate prepared from two sorbents with different selectivity for the sample. The sorbent layer for the first development is a narrow strip that abuts the much larger area used for the second development. Commercially available plates have silica gel and reversed-phase layers as adjacent zones.
 - Use a layer prepared from a mixture of two different sorbents and develop with different mobile phases such that the retention mechanism for the two, orthogonal, developments is governed by the properties of one of the sorbents in each direction.
 - After the first development the selectivity of the layer is changed by impregnation with a chemical reagent or immiscible solvent prior to the second development.
 - After the first development the properties of the sample are modified by chemical reaction or derivatization prior to the second development.
-

direction. If the same solvent is used for both developments then the sample will be redistributed along a line from the corner at which the plate was spotted to the corner diagonally opposite. In this case, only a small increase in resolution can be anticipated corresponding to the greater migration distance for the sample. The realization of a more efficient separation system implies that the resolved sample is distributed over the entire plate surface. This can be achieved only if the selectivity of the separation mechanism is complementary in the orthogonal directions. The possibility of achieving a large zone capacity for these conditions, Table 6.4, sustains interest in this technique [1,134,135].

Some potential methods for generating two different retention mechanisms in orthogonal directions are summarized in Table 6.11 [61,132,136-139]. Using two solvent systems with complementary selectivity is the simplest approach but is often only partially successful. In many cases the two solvent systems differ only in their intensity for a given set of intermolecular interactions, and are not truly complementary. Chemically bonded layers can be used in the reversed-phase and normal-phase mode and allow the use of additives and buffers as a further means of adjusting selectivity. Aminopropylsiloxane-bonded layers can be used as an ion-exchange system with acidic mobile phases and as a reversed-phase or normal-phase system with a neutral or basic mobile phase. In several reports as many as 20 to 30 components were successfully separated by two-dimensional thin-layer chromatography indicating the potential of this approach for analyzing complex mixtures, even, as seems likely, the conditions used were not truly optimum. Also, the potential increase in zone capacity obtainable by using multiple development or forced flow to minimize zone broadening and the use of solvent strength gradients, as part of the optimization strategy, seem to have largely gone unrecognized [1,61].

The acceptance of two-dimensional thin-layer chromatography for quantitative analysis rests on providing a convenient method for *in situ* detection, quantification, and

data analysis. Slit-scanning densitometers are designed for lane scanning and are not easily adapted for area scanning. Adaptation to scanning two-dimensional separations was demonstrated using normal scanning operations with small steps between scans or by zigzag scanning. Special software is required to map the layer surface and define zone locations and their optical density as three-dimensional plots or contour diagrams [61,140,141]. The awaited breakthrough in general detection for two-dimensional planar separations is likely to come from optical imaging systems (see section 6.9.4).

6.7.5 Developing Chambers

The development process in thin-layer chromatography can be carried out in a variety of chambers that differ significantly in design and sophistication [9,32,36,124]. For convenience these are often categorized as normal (N-chamber) and sandwich (S-chamber) type chambers, and further subdivided based on whether the internal atmosphere is saturated (N_S or S_S) or unsaturated (N_U or S_U). Normal chambers have a depth of gas phase in front of the layer greater than about 3 mm. For sandwich layers the gas phase is less than 3 mm. Saturation of the vapor phase is achieved by using solvent saturated pads as a chamber lining or, for sandwich chambers, a second solvent filled layer facing the separation layer. The mobile phase velocity and R_F values can be different for different chamber designs using the same stationary and mobile phases. To compare results from different chamber types, it is necessary to determine the chamber saturation grade [36].

The twin-trough chamber is the simplest of the developing chambers for conventional and high performance thin-layer chromatography [142]. It consists of a standard rectangular tank with a raised, wedge-shaped bottom. The wedged bottom divides the tank into two compartments, so that it is possible to either develop two plates simultaneously, or to use one compartment to condition the layer prior to development. The plate is placed in one compartment and mobile phase or conditioning solvent in the other. The mobile phase can be added directly to the plate compartment or, when the mobile phase is used for conditioning as well, the chamber can be tilted to allow transfer of mobile phase into the compartment for development. The twin-trough chamber is widely used for routine quality control applications.

The horizontal development chamber can be used in either the normal or sandwich configuration for either conventional edge-to-edge or simultaneous edge-to-center development [124,129,143]. Starting the development simultaneously from opposite edges enables the number of sample separated to be doubled with the same separation time. The mobile phase is transported from the reservoirs to the layer by two glass slides; the liquid rises by surface tension and capillary forces. The mobile phase then travels through the layer by capillary action. When a plate is developed from both edges simultaneously, the chamber must be leveled to allow the two solvent fronts to migrate at the same speed and meet precisely in the middle. The sandwich configuration of the horizontal developing chamber is unsuitable for mobile phases containing volatile acids or bases, or mixtures with a large concentration of a volatile polar solvent, such as

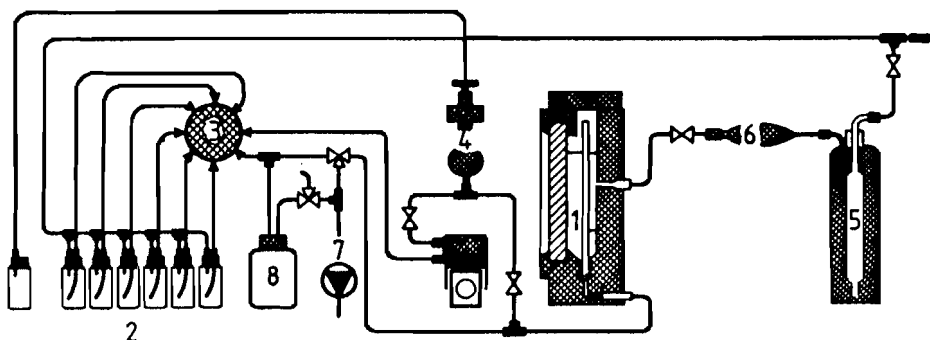


Figure 6.11. Schematic diagram of the automated multiple development chamber. Identification: 1 = developing chamber; 2 = solvent reservoirs; 3 = solvent selection valve; 4 = solvent mixer; 5 = wash bottle for preparation of the gas phase for layer conditioning; 6 = gas phase reservoir; 7 = vacuum pump; and 8 = solvent waste reservoir.

methanol or acetonitrile. The Vario-KS chamber is similar to the horizontal developing chamber [32] but allows the layer to be segregated into six individual lanes, each with its own conditioning trough and mobile phase reservoir. It allows up to six different separation conditions to be evaluated simultaneously, and is used primarily as a scouting tool to optimize separation conditions.

Automation of conventional development is possible using an automated developing chamber [9]. Preconditioning, normal or sandwich configuration, mobile phase migration distance, and the drying conditions are selectable from a microprocessor-based control unit. Mobile phase is automatically added to the developing chamber and development occurs under controlled conditions in the usual way. A sensor monitors the solvent-front migration distance. When the selected solvent-front migration distance is reached, solvent is drained from the chamber, and the layer is dried with filtered cold or warm air.

A schematic diagram of the automated multiple development chamber is shown in Figure 6.11 [61,67,124,144]. This chamber was developed for automated separations requiring incremental multiple development with a decreasing solvent strength gradient. The operating variables of layer conditioning, solvent-front migration distance, mobile phase composition for each development step, drying time, and the number of developments are entered into the computer-based control unit. Without further intervention the complete separation sequence is carried out. The layer remains in a fixed position and the mobile phase components are selected, mixed, and then delivered to the chamber. A sensor monitors the solvent-front migration distance. The development is stopped when the solvent front reaches the selected distance, mobile phase drained from the chamber, and mobile phase trapped by the layer removed by vacuum. A new development sequence is then initiated until the complete program is executed. Typically each development is 3-5 mm longer than the previous one. The first few developments with a strong solvent are used primarily to focus the sample zones.

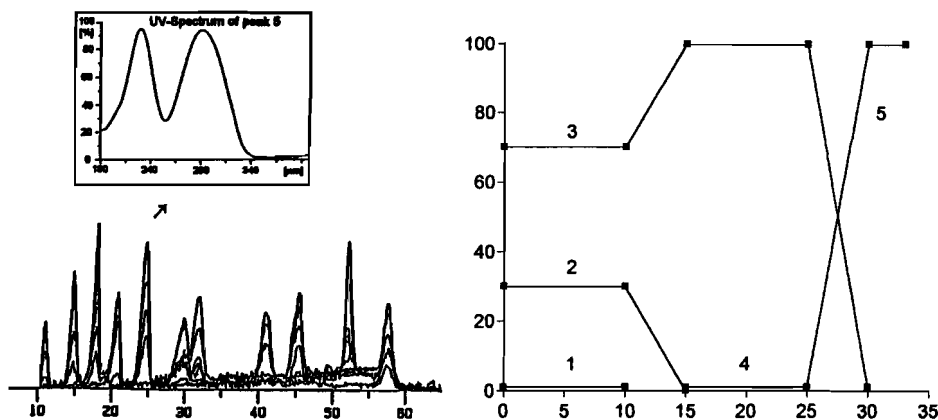


Figure 6.12. Separation of a mixture of polar crop-protecting agents on silica gel by automated multiple development. Shown are the gradient profile used for the separation, the use of multiple wavelength scanning for detection and an *in situ* UV spectra for one peak (≈ 50 ng). Solvent compositions for the gradient: 1 = aqueous ammonia; 2 = acetonitrile; 3 = dichloromethane; 4 = formic acid; and 5 = hexane. (Adapted from ref. [21]; ©American Chemical Society).

Subsequent steps are longer with weaker solvents and responsible for the separation. The number of development steps depends on the complexity of the mixture and the shape of the desired gradient profile. From 10-30 developments requiring 1.5 to 4.5 h for completion are not unusual. A typical separation of crop protecting agents employing a gradient containing both acid and base modified organic solvents, is shown in Figure 6.12 [20,21]. Screening for 265 pesticides in water with typical detection limits in the 10s of nanogram range per component (or about 50 ng / l for a 500 ml water sample prepared by solid-phase extraction) is possible. Multiple-wavelength scanning is used to improve the certainty of identification based on simultaneously matching of migration distances and absorbance ratios to those of known substances. Full *in situ* UV spectra can also be searched against appropriate libraries or matched with standards run on the same plate.

Some samples are unsuitable for separation by multiple development [67,68,71]. Compounds with significant vapor pressure may be lost during the repeated solvent evaporation steps performed under vacuum. Artifact peaks from unstable compounds may be mistaken for sample components. Compounds that are easily oxidized, photolyzed, or hydrolyzed to more polar products yield two separated zones in each development. In a multi-step program, the accumulative effect can result in a complex chromatogram from a single component. Solvents of low volatility and/or high polarity should be avoided, since they are only slowly removed from the layer by vacuum and considerably extend the total separation time. Solvent impurities can be a source of ghost peaks (identifiable by scanning between sample lanes).

Two general approaches are used for forced flow separations. Rotational planar chromatography uses centrifugal forces created by spinning the plate around a central

axis to move the mobile phase through the layer [31,42]. The rotation speed can be varied from 80 to 1,500 rpm. By scrapping out zones in the layer, either circular, anticircular or linear development is possible. A collection system fixed to the rotor allows separated fractions to be collected as they leave the layer when operated in the circular mode for preparative-scale applications. The overpressured development chamber eliminates the vapor space above the layer by contacting the layer surface with a plastic membrane pressurized on its opposite surface by hydraulic pressure of up to 50 atmospheres [12,45,46]. A mechanical pump forces the mobile phase through the layer at the selected velocity. Special layer preparation is required for linear development. The sorbent is removed from the bottom (sample application end) and two sides of the layer to a distance of about 5 mm and the edges sealed with latex glue [48]. This ensures that the mobile phase flow occurs only in the desired direction. An eluent trough scored into a polymeric sheet inserted between the hydraulic cushion and the layer is used to create a linear solvent front. A second insert at the exit position is required if the chamber is used in the elution mode with on-line detection, similar to normal operation in column liquid chromatography. Since only a single sample can be separated with on-line detection, this is normally used to identify fractions for collection in preparative-scale chromatography or coupling to other separation or identification techniques.

6.8 METHOD DEVELOPMENT

Since the zone capacity in thin-layer chromatography is limited (Table 6.4), the number of detectable components in the mixture and their range of polarity is used to determine the development technique. For capillary flow separations, in a single development, mixtures containing more than 8 to 10 components may be too difficult or impossible to separate. In addition, if the range of polarities is too wide then multiple development techniques using a mobile phase gradient will be necessary. Of course, all components of a mixture may not be of equal interest and then the goal of the method is to separate the components of major interest from each other and from the less important components as a group. Method development will be aided if standards for the relevant compounds are available. Standards simplify zone tracking and enable detection characteristics and the possibility of spectroscopic resolution of incompletely separated zones to be established. Standards are also required for calibration, if quantification is required, and to construct spectral libraries for identification purposes. The expected concentration range of relevant compounds may indicate the need for derivatization to obtain required detection limits.

A general guide to stationary phase selection for separations by thin-layer chromatography is summarized in Figure 6.13 (see section 6.4). Silica gel is the most widely used stationary phase for thin-layer chromatography and is generally the first choice for the separation of low molecular mass organic compounds soluble in common organic solvents. Most thin-layer sorbents are small pore materials optimized for the separation of compounds with molecular weights below about 750. Precipitation chromatography is

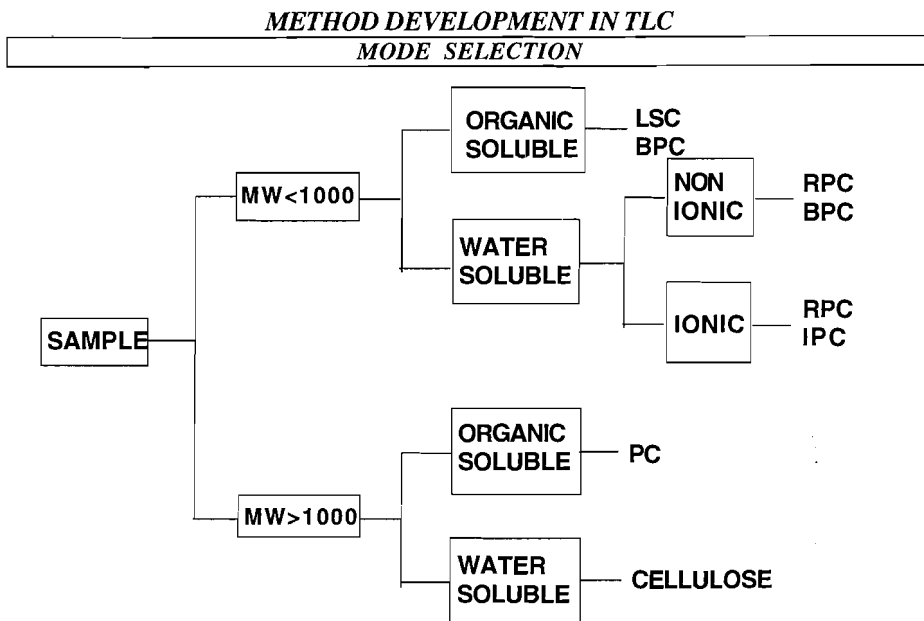


Figure 6.13. Separation mode selection guide for TLC. LSC = liquid-solid chromatography on an inorganic oxide adsorbent; BPC = liquid-solid chromatography on a chemically-bonded sorbent; RPC = reversed-phase chromatography with a water-containing mobile phase and chemically-bonded stationary phase; IPC = ion-pair chromatography with reversed-phase separation conditions; and PC = precipitation chromatography. (From ref. [151]; ©Elsevier)

a unique application of thin-layer chromatography to the separation of high molecular mass polymers based on solubility differences in a mobile-phase composition gradient [145,146]. For the separation of water soluble, high-molecular-mass biopolymers cellulose layers are about the only useful choice. Reversed-phase chromatography on chemically bonded layers is generally used for the separation of compounds difficult to separate on silica gel, Figure 6.14. Compounds of low polarity can be difficult to separate on silica gel because of weak retention (mobile phase selection is limited because most solvents are too strong for these separations) and polar compounds because of strong retention (mobile phase selection is limited because most solvents are too weak for these separations). Ions and easily ionized compounds are frequently separated by reversed-phase chromatography using buffered mobile phases (weak acids and bases) or ion-pair reagents (strong acids and bases). There are only a limited number of stationary phases suitable for ion-exchange chromatography. Except for 3-aminopropylsiloxane-bonded layers, which function as a weak anion exchanger with an acidic mobile phase, ion exchange is not a widely used separation mechanism in thin-layer chromatography.

Given the similarity of retention mechanisms, it is hardly surprising that the principal methods of mobile phase selection in thin-layer chromatography are similar to those

Silica Gel General Adsorption Scale

Difficult to separate because solvent strength is too high	Alkanes	Weak ↓ Strong
	Aromatics	
	Halogenated Compounds	
	Ethers	
	Nitro Compounds	
	Nitriles	
	Carbonyl Compounds	
	Alcohols	
	Phenols	
	Amines	
	Amides	
Difficult to separate because solvent selectivity is too low	Carboxylic acids	
	Sulfonic acids	

Figure 6.14. General adsorption scale for separations by silica gel thin-layer chromatography.

followed in column liquid chromatography (section 4.4.1). Since the mobile phase is evaporated prior to detection, a wider range of UV absorbing solvents are commonly used in thin-layer chromatography than is the case for column liquid chromatography. Solvents must be of high purity, since involatile impurities and stabilizers intentionally added to some solvents, remain adsorbed to the layer causing sloping and unstable baselines in scanning densitometry [86]. The most significant difference between column and thin-layer methods is that in thin-layer chromatography equilibrium is not necessarily established throughout the separation. When multi-component mobile phases are used, composition gradients can form in the development direction due to demixing (see section 6.3.2). Demixing effects are less apparent when saturated developing chambers are used. The presence of a vapor phase in TLC further complicates matters with both migration distances and migration order influenced by the level of saturation of the development chamber [49]. These considerations hinder optimization strategies based on the composition of the solvent added to the developing chamber as popularized in column liquid chromatography.

The selection of a mobile phase for the separation of simple mixtures may not be a particularly difficult problem and can be arrived at quite quickly by guided trial and error experiments [9,147-152]. A mobile phase with the correct strength for a single development will migrate the sample into the R_F range 0.2-0.8, or thereabouts. If of the correct selectivity, it will distribute the sample components evenly throughout this range, or meet some other resolution criterion established for the particular separation. Solvent systems can be screened in parallel using either several development

chambers or a device like the Vario-KS chamber (section 6.7.5). Alternatively, sample spots (up to 16 on a high performance layer) are applied at suitable positions on a single layer and automatically developed in sequence with 45 μ l of solvent or solvent mixtures using an automated sample applicator [150,153]. The individual circular chromatograms enable rapid identification of solvents with suitable strength and selectivity for the separation. The three-point window diagram [153] and simple mixture design approaches [150,154,155] are other methods suitable for the quick identification of separation conditions for simple mixtures. However, whenever the number of components in a mixture exceeds all but a small fraction of the zone capacity for the separation system, a more systematic method of mobile phase optimization is required.

6.8.1 Prisma Model

The Prisma model is widely used for optimization of mobile phase composition in thin-layer chromatography [156,157]. Application of the model proceeds in three stages: (i) selection of the chromatographic system, (ii) optimization of the selected mobile phase components, and (iii) selection of the development method. The optimization procedure starts with silica gel, since this is the most commonly used stationary phase for thin-layer chromatography, although the method is equally applicable to normal-phase and reversed-phase separations on chemically bonded layers. Suitable solvents for the separation are identified in an initial experimental screening step. These experiments are carried out on thin-layer plates in unsaturated chambers with single solvents chosen from the different selectivity groups according to Snyder (Table 4.14, section 4.4.1). After these initial experiments, the solvent strength is adjusted so that the sample components are distributed in the R_F range 0.2-0.8. If the substances migrate into the upper third of the plate the solvent strength is reduced by dilution with hexane, the strength adjusting solvent (solvent strength = 0). If the substances remain in the lower third of the plate with the single solvents their solvent strength has to be increased by addition of a strong solvent such as water or acetic acid. A similar procedure is followed for reversed-phase chromatography using water miscible solvents and water as the strength adjusting solvent (solvent strength = 0). From these trial experiments, mobile phases giving the best separation (maximum number of separated zones) are selected for further optimization in the second part of the model. If it is anticipated that forced flow will be used for the separation then it is advantageous to include one solvent in the selected solvent systems in which the sample does not migrate. This solvent can be used in a prerun to eliminate the disturbing zone caused by undissolved gases in the sealed layer (section 6.3.3).

Between two and five solvents can be selected for mobile phase optimization. Modifiers such as acids, ion-pair reagents, etc., can be added to improve the separation and reduce tailing. Modifiers are generally used in a low and constant concentration so that their influence on solvent strength can be neglected. The Prisma model, Figure 6.15, is a three dimensional geometrical design which correlates the solvent strength with

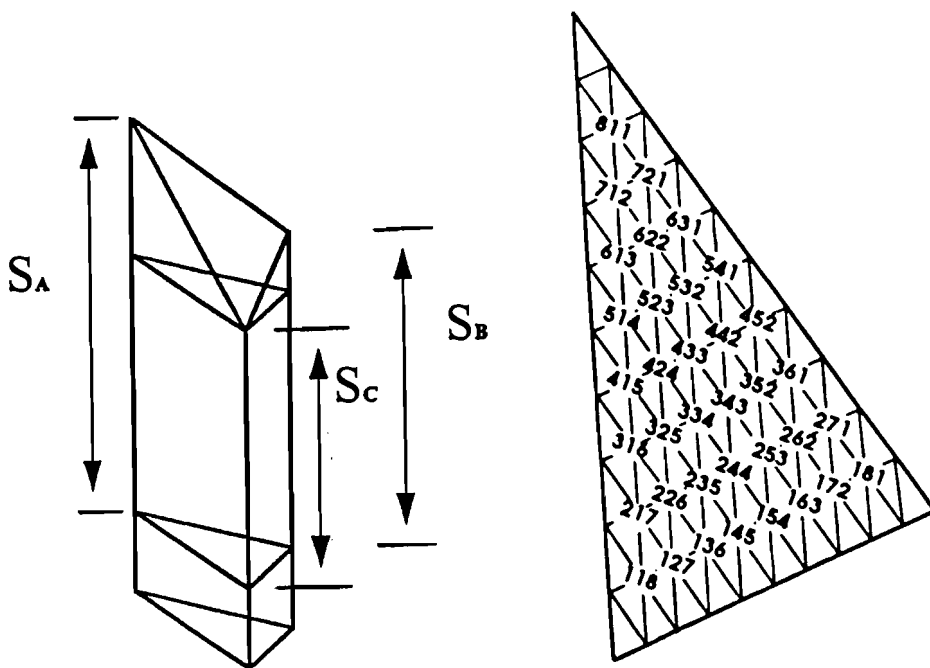


Figure 6.15. The Prisma mobile phase optimization model showing the construction of the prism and the selection of selectivity points.

the selectivity of the mobile phase [156]. The model consists of three parts: the base or platform representing the modifier; the regular part of the prism with congruent base and top surfaces; and the irregular truncated top prism (frustum). The lengths of the edges of the prism (S_A , S_B , S_C) correspond to the solvent strengths of the single solvents (A, B and C). Since the selected solvents usually have different solvent strengths, the edges of the prism may be of unequal length and the top plane of the prism will not be parallel and congruous with its base. Cutting the prism parallel to its base at the height of the lowest edge (determined by the solvent strength of the weakest solvent, solvent C in Figure 6.15), gives a regular prism, where the top and any planes representing weaker solvents diluted with a strength adjusting solvent are parallel equilateral triangles. The upper frustum is used for mobile phase optimization of polar compounds in normal-phase chromatography, while the regular part is used for the separation of non-polar and moderately polar substances. For reversed-phase chromatography, the regular part of the prism is used to optimize the separation of both non-polar and polar substances.

For polar compounds optimization is always started on the top irregular triangle of the model, either within the triangle, when three solvents are selected, or along one side, for binary mobile phases. Any solvent composition on the face of the triangle can be

represented by a three-coordinate selectivity point (P_S); each coordinate corresponding to the volume fraction of the solvent at that position on the triangle, Figure 6.15. Optimization is commenced by selecting solvent combinations corresponding to the center point $P_S = 333$ and three other points close to the apexes of the triangle $P_S = 811$, 181 and 118. If the separation obtained is insufficient other selectivity points are tested around the solvent combination that gave the best separation. On changing the selectivity points on the top triangle the solvent strength changes as well, especially when the solvent strengths of the solvents used to construct the prism are considerably different. The strength of the solvent should be adjusted with the strength adjusting solvent to maintain the separation in the optimum R_F range. It may also be advisable to change the selectivity points by small increments if regular step sizes cause large changes in resolution. To aid optimization experimental data can be fitted to a 3-dimensional retardation surface with x- and y-coordinates as selectivity points and the z-coordinate as the R_F value [158,159].

The regular center portion of the prism is used to optimize the mobile phase composition for the separation of non-polar and moderately polar compounds. The initial solvent composition corresponds to the center of the triangular top face of the regular prism ($P_S = 333$); this composition is then diluted to bring all sample components into the R_F range 0.2-0.8. At this solvent strength three more chromatograms are run corresponding to the selectivity points close to the apexes of the triangle. These initial runs are then used to choose selectivity points for further chromatograms until the best solvent composition is located. For saturated developing chambers there is a linear relationship between R_F values and the solvent strength at a constant P_S value ($\ln R_F = d(S_T) + e$, where S_T is the solvent strength and d and e are regression constants). At a constant solvent strength there is a quadratic relationship between the R_F value and the selectivity points describing the retardation surface [$R_F = a (P_S)^2 + b (P_S) + c$, where a , b and c are regression constants). These relationships can form the basis of a computer-aided optimization strategy according to a fixed experimental design requiring 18 experiments [152] or a general approach with decisions based on the interpretation of the retardation surfaces [159,160]. Within the concept of the Prisma model, selection of the correct solvent strength is referred to as vertical optimization, and identification of the optimum mobile phase composition at a fixed solvent strength as horizontal optimization.

Optimization of the solvent strength by varying the selectivity points is carried out until the required separation is obtained. If no adequate separation is obtained then a different layer or additional solvents must be selected and the new system optimized by the previous procedure. Nearly adequate separations can be improved in the third part of the Prisma model by selecting a different development mode. If an increase in efficiency is required to improve the overall separation then forced flow methods should be used. If the separation problem exists in the upper R_F range then anticircular development may be the best choice, if in the lower R_F range, then circular development is favored.

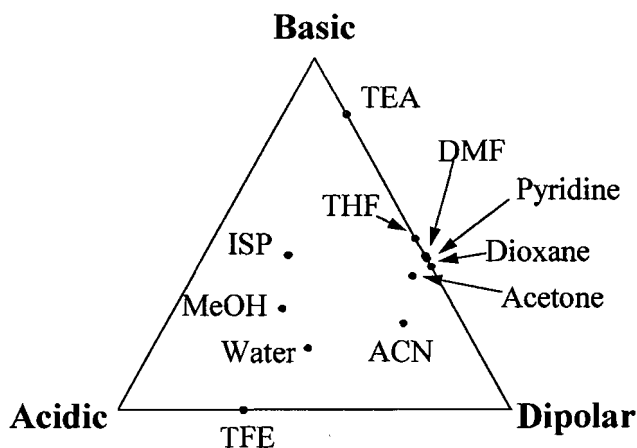


Figure 6.16. Solvent-selectivity triangle based on normalized solvatochromic parameters for some common water miscible organic solvents. TEA = triethylamine, THF = tetrahydrofuran, DMF = N,N-dimethylformamide; ISP = 2-propanol; MeOH = methanol; ACN = acetonitrile; and TFE = 2,2,2-trifluoroethanol. (From ref. [95]; ©Research Institute for Medicinal Plants).

6.8.2 Solvation Parameter Model

The solvation parameter model has been used as the basis of a structure-driven retention model for method development in reversed-phase thin-layer chromatography [95,151,161-164]. The model should be applicable to normal-phase separations on chemically bonded layers as well, but not silica gel layers [164]. Solute size differences and site-specific interactions on silica gel are not adequately accounted for by the model, which results in poor predictions of retention. The solvation parameter model is described in section 1.4.3. The R_M value (section 6.3.1) is used as the dependent variable.

The selection of mobile phase components in reversed-phase chromatography is restricted to solvents that are miscible with water. These solvents can be classified according to their capability for different intermolecular interactions based on their solvatochromic parameters (section 4.4.1). A visual indication of solvent selectivity differences is obtained by plotting the solvents on the surface of a triangle using normalized coordinates, Figure 6.16 [95]. The most selective solvents are found along the edges and towards the apexes for the indicated interactions with those solvents containing a blend of properties located on the face. The selectivity range that can be achieved with the three common solvents methanol, acetonitrile, and tetrahydrofuran is rather limited as indicated by their position on the triangle compared to the greater selectivity space available if all solvents are considered. It is also clear from the distribution of solvents on the triangle that solvents, which are strong hydrogen-bond bases with weak dipole-type properties, are uncommon. Triethylamine is the best of these solvents but is immiscible with water and could only be used as a component of a ternary mobile phase containing a polar organic solvent. Acetone, acetonitrile, 2-

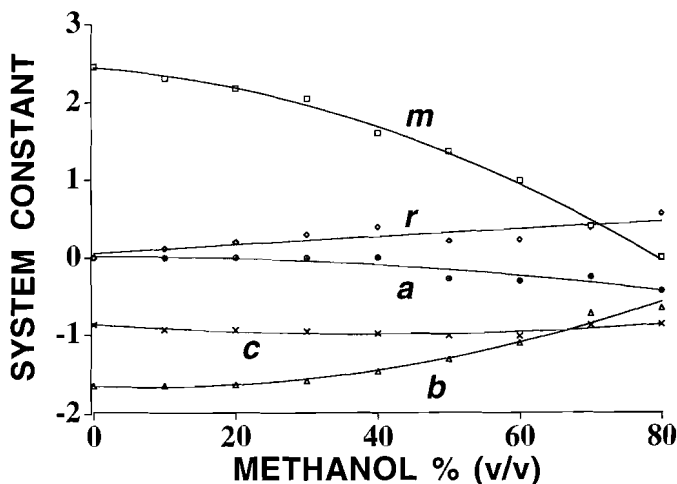


Figure 6.17. A system map for a cyanopropylsiloxane-bonded layer with methanol-water as mobile phase. The m system constant is a measure of the difference in cohesion and dispersion interactions between the solvated layer and the mobile phase; the r system constant the difference in capability for electron lone pair interactions; the a system constant the difference in hydrogen-bond basicity; the b system constant the difference in hydrogen-bond acidity; c is the model constant and among other contributions contains the phase ratio; and the s system constant representing differences in dipole-type interactions is zero at all mobile phase compositions.

propanol, methanol and 2,2,2-trifluoroethanol provide a convenient range of hydrogen-bond acidity. Methanol and 2-propanol are simultaneously strong hydrogen-bond bases and dipolar, and for this reason are found towards the center of the triangle. They could be considered as polar solvents of low selectivity. 2,2,2-Trifluoroethanol is the strongest hydrogen-bond acid (stronger than water) and has zero hydrogen-bond basicity. It is quite dipolar but considerably more selective than the other alcohols for hydrogen-bond interactions. Acetonitrile (or dioxane), acetone (or tetrahydrofuran), and pyridine (or N,N-dimethylformamide) provide a reasonable range of hydrogen-bond base properties. Acetonitrile is the most dipolar solvent with minimal (although significant) capacity for hydrogen-bond interactions. The six water miscible organic solvents (acetone, acetonitrile, 2-propanol, methanol, 2,2,2-trifluoroethanol and pyridine) are expected to provide a reasonable range of separation selectivity for reversed-phase separations.

Method development for binary mobile phases using the solvation parameter model is based on the use of system maps. A system map is a continuous plot of the system constants obtained from experimental data fit to the solvation parameter model against mobile phase composition. However, once constructed the system map is a permanent record of system properties. It is used in all calculations and is not restricted to the compounds used to construct the system map. A typical system map for methanol-water mobile phases on a 3-cyanopropylsiloxane-bonded layer is shown in Figure 6.17. System maps for several binary mobile phases on octadecylsiloxane-bonded [95],

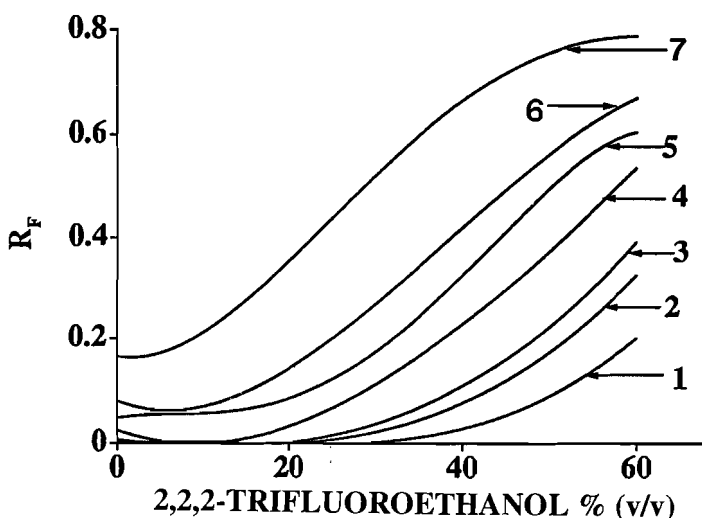


Figure 6.18. Retention map for the separation of analgesics by reversed phase thin-layer chromatography on an octadecylsiloxane-bonded layer with 2,2,2-trifluoroethanol-water mixtures as mobile phase. Compounds: 1 = chloropheniramine; 2 = ibuprofen; 3 = naproxen; 4 = phenacetin; 5 = aspirin; 6 = caffeine; and 7 = acetaminophen.

cyanopropylsiloxane-bonded [162], and spacer bonded propanediol [151] layers are available.

Retention maps are created from the system maps for all compounds to be separated. This is conveniently done using a spreadsheet for the calculations and graphics for evaluation. All mobile phase and stationary phase combinations for which system maps are available can be compared in the search for the optimum system. A typical retention map for the separation of a mixture of analgesics on an octadecylsiloxane-bonded layer with 2,2,2-trifluoroethanol-water as a mobile phase is shown in Figure 6.18 [95]. Those solvent compositions resulting in acceptable zone separation are easily identified by visual inspection. Computer simulation of retention maps allows those systems likely to provide an acceptable separation to be identified before experimental work commences. Consequently, this approach is able to direct experimental procedures and not just simply aid in their interpretation. The agreement between model predicted and experimental R_F values is generally good. Differences are typically less than 0.05 R_F units. Retention surfaces for the optimization of ternary solvent systems based on a mixture-design approach combined with the solvation parameter model have also been demonstrated [164].

6.8.3 Computer Simulations

The solvation parameter model (section 6.8.2) can be considered a suitable model for computer-aided method development. Computer-aided strategies for mobile phase

optimization using window diagrams, overlapping resolution maps, simplex methods, and iterative procedures have found occasional use in thin-layer chromatography, but not to the same extent as for column chromatography [9,147-149,151,165]. In these procedures, some form of statistical design is used to select a group of solvents for evaluation, or alternatively, the results obtained from an arbitrary selection of solvents are compared to indicate the best separation. In the simplex method an evolving experimental design is used to predict new mobile phase compositions from initial solvent compositions guided by a set of rules that (hopefully) direct the movement of the simplex to a solvent composition providing an acceptable separation. To rank separation quality for computer interpretation a single-value numerical index, or a technique, such as overlapping resolution maps for visual evaluation, is used [9,166]. Many mathematical functions are used to characterize the quality of thin-layer separations but none can be considered ideal. The choice of the separation quality index can lead to the prediction of different optimum mobile phase compositions for a separation, which is disconcerting. Resolution based functions favored in column chromatography (section 1.6.2) are not useful in thin-layer chromatography because zone widths are difficult to predict and depend on migration distance. Typically, separation quality is defined by an index based on the zone center separation, sometimes combined with a function to characterize the zone distribution throughout the separation.

Computer simulation of separations for binary mobile phases containing a single strong solvent are usually based on one of the functions $R_M = a \log(X_S) + b$ or $R_F = a(X_S)^2 + b(X_S) + c$ where a , b and c are regression constants and X_S is the mole fraction of strong solvent [167]. A certain amount of experimental data is required to determine the regression coefficients after which additional R_F values can be estimated by interpolation. This approach can only estimate results for compounds included in the initial experiments. Results for simulation of two-dimensional separations based on sequential application of the above equations for one-dimensional separations varied from poor to reasonable without obvious reasons for the variation [61,168,169].

6.8.4 Gradient Methods

Mobile phase composition gradients are required for the separation of samples containing components of a wide retention range. Stepwise solvent strength gradients are easier to implement than continuous gradients in thin-layer chromatography, especially when multiple development is used for the separation (section 6.7.3). Soczewinski's group described a modification of the horizontal developing chamber for stepwise gradient development with increasing solvent strength mobile phases [170,171]. The modified design of the solvent delivery system allows the mobile phase to be adsorbed to the last drop. Therefore, successive volumes of mobile phase of increasing solvent strength can be introduced after complete adsorption of the previous fraction by the layer. The total of all volume fractions of each mobile phase used to construct the gradient is usually equivalent to the hold-up volume of the layer. Solvent consumption is minimal and any gradient program, including continuous or multiple

component gradients can be generated. The real gradient profile in the layer, however, may be different to the programmed gradient due to solvent demixing and the slow exchange of stagnant mobile phase in the sorbent pores with the mobile phase of higher solvent strength.

Multiple gradient development (MGD) employs successive development of the layer over decreasing distances with mobile phases of increasing solvent strength [172,173]. The layer is dried between developments. Each development distance is selected such that the position of the most advanced zones separated in the first few developments remain fixed in position by locating the new solvent front at a level below them. The method is easily performed in the horizontal developing chamber with minimal solvent use. The most important approach to stepwise gradient development uses multiple development over increasing migration distances with a decreasing solvent strength gradient. This is the basis of the automated multiple development chamber (section 6.7.3).

Suitable models for the calculation of migration distances in stepwise gradient development using eluent fractions of increasing solvent strength without interruption of the development process [171,174] and by incremental multiple development with increasing and decreasing solvent strength gradients [172,173,175-177] have been described. The general approach is similar in all cases. The relationship between the R_F value and mobile phase composition for each mobile phase used to construct the gradient is determined experimentally and fit to a mathematical function as described in section 6.8.3. For computer simulation, the number of developments and the composition of the mobile phase and solvent-front migration distance for each development are selected. The computer then calculates the migration distance for each component by considering each development step separately and updating the zone origin for each component based on its location in the previous development. Computer simulation enables the influence of solvent composition, number of developments and solvent-front migration distance to be optimized before attempting the separation. A significant number of isocratic experiments are required to establish the relationship between solvent composition and analyte migration distance. Agreement of simulated separations with experiment are likely to be poor when the relationship between mobile phase composition and migration distance is not well determined (errors tend to accumulate through each step in the program). The predictive accuracy is also impaired if sorbent properties are modified by the solvents used in the previous development and by solvent demixing.

Optimized gradients for automated multiple development are usually arrived at by pragmatic means rather than computer simulation [9,61,67,104,178,179]. Two general approaches have been adopted for guided trial and error procedures. The first is based on the use of a universal gradient, which commences with methanol, ends with hexane, and uses dichloromethane or methyl *tert*-butyl ether as the intermediate or base solvent (typically employing 25 steps) [104]. By scaling and superimposing the chromatogram of the separation above the theoretical gradient profile, those regions of the chromatogram affecting the separation are easily identified. The appropriate

initial mobile phase composition for the first development and the last development are easily identified, thus eliminating steps in the program that are not contributing to the separation. The gradient shape can then be modified to enhance resolution in those regions of the chromatogram that are poorly separated or to make better use of the zone capacity by minimizing regions devoid of sample zones. Thus, for example, the program can be modified to provide a shallower gradient over those regions of the chromatogram where peak separation is inadequate and steeper gradients in regions where the separated zones are well displaced from each other. For relatively simple mixtures this approach is often satisfactory. The universal gradient is not a linear solvent strength gradient, however, and the abrupt changes in solvent strength during the gradient can result in poor resolution by crowding sample components into a limited space [180].

Resolution may be inadequate in automated multiple development because of zone distortion, particularly tailing. This can be controlled by adjusting the layer conditioning step, or adding a tailing inhibitor in low concentration (e.g. formic acid, water, ammonia, etc.) to those compositions that are responsible for migration of the distorted zones. However, in those cases where the resolution remains inadequate after making the above adjustments, it is necessary to identify a different gradient composition for the separation. The Prisma model (section 6.8.1) can be used to identify more selective solvents to replace the initial, terminal, or base solvent; or for use in those program segments that affect the separation of poorly resolved zones in the universal gradient.

Alternatively, if the composition of the sample is known, and standards are available, isocratic plots of R_M (section 6.3.1) against mobile phase composition can be used to infer suitable gradient separation conditions [181,182]. The initial solvent is chosen such that it possesses sufficient strength to cause migration of the most retained components of the mixture. The final solvent is selected to provide an acceptable separation of the least retained components without migrating them too close to the solvent front. The base solvent is selected based on its ability to provide optimum zone spacing throughout the chromatogram.

6.9 DETECTION

At best, inspection by eye of a thin-layer plate is capable of detecting about 1-10 micrograms of colored substances with reproducibility rarely better than 10-30%. Excising separated zones, eluting the substance from the sorbent, and determining the analyte concentration in solution by spectrophotometry is time consuming and often inaccurate. Difficulties in accurately locating the spot boundary by eye, incomplete elution of the sample from the sorbent and non-specific background absorption due to colloidal particles in the analytical solution are the main reasons for these deficiencies. Instruments for *in situ* recording and quantification of thin-layer chromatograms first appeared in the mid-1960s and are now considered essential for routine quantitative analysis in thin-layer chromatography [8,9].

All optical methods for the quantitative evaluation of thin-layer chromatograms are based on determining the difference in optical response between a sample free region of the layer and regions of the layer where sample components are present [9,11,183-186]. Transmission measurements can be used but reflectance is more common. In the reflectance mode most of the scattered light arises from particles close to the surface and is influenced less by variations in the layer thickness, which is responsible for much of the background noise in transmission measurements. Transmission measurements are limited to wavelengths greater than 320 nm due to strong absorption by the glass backing plate and by silica gel itself at shorter wavelengths. Reflectance measurements can be made at any wavelength from the UV to the near infrared (185-2500 nm).

When monochromatic light falls on an opaque medium some light is reflected from the surface, some absorbed by the medium and dissipated in some way, such as by conversion to heat, and the remainder is diffusely reflected or transmitted by the medium. For quantification it is the diffusely reflected/transmitted light that is of importance. The specularly reflected component only contributing to detector noise and not to the signal. The propagation of light within an opaque medium is a complex process that can only be solved mathematically if simplifying assumptions are made [9,11,184,186-189]. The most generally accepted theory is due to P. Kubelka and F. Munk, who proposed several solutions, the simplest being

$$(1 - R_{\infty})^2 / 2 R_{\infty} = 2.3 (aC / S) \quad (6.14)$$

where R_{∞} is the reflectance for an infinitely thick opaque layer, a the molar absorption coefficient of the sample, C the sample concentration, and S the scatter coefficient for the layer. Equation (6.14) is derived explicitly for a layer of infinite thickness, which is not an accurate representation of separation layers, and this equation can only provide a phenomenological model for absorption measurements. However, it serves to illustrate the general properties of a solid-sorbent matrix on the observed sample response, and in a simple way, explains why calibration curves obtained on thin-layer plates do not obey Beer-Lambert's Law. It predicts, for example, a nonlinear relationship between signal and sample concentration in the absorption mode, an increase in response with larger molar absorption coefficients, and an increase in response for sorbents having a low scatter coefficient.

The absence of a suitable model for the relationship between absorption and sample concentration results in calibration being the principal method for quantification in thin-layer chromatography. Calibration curves are individual in shape, often pseudolinear at low sample concentrations curving towards the concentration axis at higher concentrations, and eventually reaching an asymptotic value where signal and sample concentration are no longer correlated, Figure 6.19. The pseudolinear region may be sufficiently wide for calibration for some compounds, but more generally nonlinear calibration is used based on a second order polynomial fit for calibration standards spanning the range of sample concentrations. Samples are quantified by interpolation only. A number of linearization methods have been proposed for calibration, but these

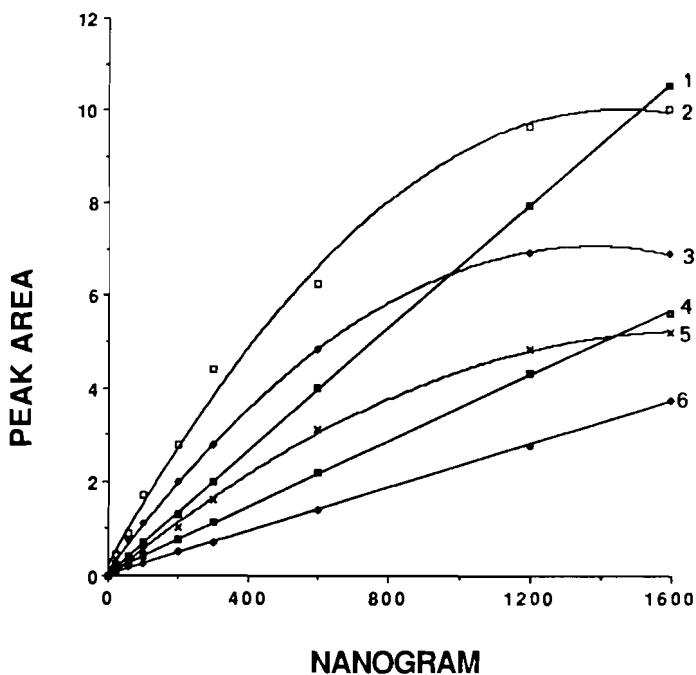


Figure 6.19. Typical calibration curves for different substances measured by absorption in the reflectance mode. Identification: 1 = practolol; 2 = azobenzene; 3 = diphenylacetylene; 4 = alprenolol; 5 = estrone; and 6 = pamatolol. (From ref. [173]; ©Elsevier).

seem both unnecessary and inappropriate, since they treat incorrectly the transposition of random errors in the data [183,184].

The fluorescence quenching technique is used to visualize sample zones that absorb UV-light on thin-layer plates containing a fluorescent indicator compound. When such a plate is exposed to UV light, UV-absorbing zones appear dark against a brightly fluorescing background of a lighter color. The UV-absorbing sample zones behave like an optical filter, absorbing a portion of the fluorescence excitation energy, which consequently diminishes the fluorescence emission intensity originating from the sample zone. The fluorescence quenching method is only applicable to substances that have an absorption spectrum that overlaps the excitation spectrum of the fluorescent indicator. Fluorescent indicators in common use have an absorption maximum around 280 nm and virtually no absorption below 240 nm. Fluorescence quenching is a less specific and less sensitive method of quantification than absorption measurements. Its main use is as a visualization technique for qualitative analysis.

Fluorescence measurements are fundamentally different to absorption measurements on thin-layer plates [184,190]. The fluorescence emission at low sample concentrations, F , is adequately described by $F = \phi I_0 abC$ where ϕ is the quantum yield, I_0 the intensity

of the excitation source, a the molar absorption coefficient, b the layer thickness, and C the sample amount. Except for the sample amount, all terms in this expression are constant, or fixed by the experiment. Consequently, the fluorescence emission is linearly related to the sample amount. Calibration curves in fluorescence are usually linear over two or three orders of magnitude. For large sample sizes, self-absorption becomes a problem, and the fluorescence signal curves towards the concentration axis. The two-point calibration method provides an alternative calibration procedure that sustains sample throughput in screening studies when several components require quantification [191,192]. This method requires only a single standard concentration for each substance, and only a single lane for all calibration standards. The method is based on the linear relationship between slit width and fluorescence intensity in slit-scanning densitometry, and the linear relationship between this slope and sample amount. Thus, in practice, scanning each sample and the lane containing standards with two slit widths (e.g. 0.4 and 0.8 mm) is all that is required for calibration. The two-point calibration method is not a replacement for standard calibration procedures when the highest accuracy is required. It is used primarily to determine approximate sample amounts in large-scale screening programs.

6.9.1 Derivatization Techniques

There is a long history of using chemical reactions for the visualization of colorless compounds in thin-layer chromatography [5,6,11,193-195]. A primary reasons for employing thin-layer chromatography in analysis is the ease and flexibility of applying selective chemical reactions for the quantification of substances with poor UV-absorption characteristics and to obtain additional selectivity for functional group and family type detection. Different color-forming reactions combined with the measurement of migration distance in separation systems with complementary selectivity are widely used in forensic and environmental surveillance programs for the identification of drugs of abuse, pesticides, preservatives, etc. [13-15,196]. The combined information from chemically-selective reactions and selective chromatographic separations results in more certain identification of an unknown residue from many hundreds or thousands of possibilities in less time than the use of chromatographic techniques alone.

Prechromatographic reactions are favored when it is desired: to modify the properties of the sample to improve stability (i.e. minimize oxidative and catalytic degradations during measurements); to optimize chromatographic resolution; or to simplify the optimization of the reaction conditions [101,190,193]. On the other hand excess reagent, reaction by-products, catalysts used for the reaction, etc., may interfere with the detection of sample components because of their location and/or intensity in the chromatogram. In addition, some mixtures may be more difficult to separate because structural differences are minimized.

The advantages of postchromatographic reaction methods are that the separation is unaffected by the reaction, by-products of the reaction do not interfere in the chromatogram, and all samples and standards are derivatized simultaneously and

with the same conditions. The reagent and derivative must have different detection characteristics, however, and the reagent must be evenly distributed over the layer. Since both prechromatographic and postchromatographic methods enhance the sensitivity and selectivity of the detection process, choosing between them will usually depend on the chemistry involved, ease of optimization, and which method best overcomes matrix and reagent interference.

For postchromatographic reactions the reagents can be applied to the layer through the gas phase or by evenly coating the layer with a solution of the reagents. Gas phase methods are fast and convenient but restricted by the number of useful reagents. Examples include iodine, ammonia, hydrogen chloride, etc., which are applied by inserting the layer into a tank containing a saturated atmosphere of the reactive vapor. Fluorescence can be induced in a number of compounds by heating them in a sealed chamber containing an activating reagent such as ammonium bicarbonate or zirconyl sulfate [197]. The mechanism of this reaction remains a mystery and results vary widely for different compounds, but for some applications, it is useful.

Spraying, dipping or contact with a sponge soaked in reagent solution is used to apply reactive compounds in solution to the layer [194,198,199]. Spray techniques using simple atomizers have a long history of use in thin-layer chromatography, but reagent application by this method is difficult to perform well. The homogeneity of the reagent distribution over the layer depends on many factors such as droplet size, distance between the spray device and layer, direction of spraying, and discharge rate of the reagent. Manual spraying is not easy to control and if ventilation of the workspace is inadequate, it can be a health hazard. For quantitative methods, controlled immersion of the layer into a solution of the reagents is the preferred technique, since it does not rely on manual dexterity and produces superior results in scanning densitometry. Some solutions do not make good dipping solutions because they contain solvents that are too aggressive or viscous for convenient application (e.g. aqueous strong acids and bases). Dipping solutions are usually less concentrated than spray solutions and water is often replaced by an alcohol for adequate permeation of reversed-phase layers. In general, it is necessary to reformulate dipping solutions from earlier recipes for spray solutions, as well as possibly making changes to the reaction conditions [193,200]. Automated low volume dipping chambers are now available and are preferred for obtaining a uniform speed and dwell time for the immersion process. Immersion typically requires only a few seconds, which is long enough to impregnate the layer with solution but not long enough to wash sample components off the layer. In the absence of automation, dipping techniques share some of the problems inherent with spraying, in that the results depend on manual dexterity.

Postchromatographic derivatization reactions can be classified as reversible or destructive depending on the type of interaction the reagents undergo with the separated compounds, and as selective or universal, based on the specificity of the reaction. The most common reversible methods employ iodine vapor, water, fluorescein, or pH indicators as visualizing reagents [193-95,201]. In the iodine vapor method, the dried plate is enclosed in a chamber containing a few crystals of iodine; substance zones are

stained more rapidly than the layer and appear as yellow-brown spots on a light yellow background. As little as 0.1 to 0.01 μg of sample can be visualized in this way. Simply removing the plate from the visualization chamber and allowing the iodine to evaporate reverses the reaction. Spraying the layer with water reveals hydrophobic compounds as white spots on a translucent background if the water-moistened layer is viewed against a white light. Solutions of pH indicators (e.g. bromocresol green, bromophenol blue, etc.) are widely used for the detection of acidic and basic compounds. The above methods are all fairly universal and reversible, and can be used for detection when the sample is required for further studies. Occasionally these methods are also used for quantitative analysis.

Irreversible methods are common for quantification and comprise hundreds of reagents based on selective chemistries reduced to standard operations over several decades of use [11,193]. A typical example is the use of copper sulfate or manganese chloride in phosphoric acid solution for the quantitative analysis of lipids. After spraying or dipping in the acid solution and heating, the lipids are converted to colored zones with a nearly uniform response [202]. Functional group specific or compound class selective reagents are used for the determination of low levels of substances in complex matrices, such as biological fluids and plant extracts.

6.9.2 Sorbent-Aided Response Modification

The fluorescence emission for substances in thin-layer chromatography are at times less than expected when predicted from solution experiments, occur at different excitation and emission wavelengths compared with solution spectra, and may produce a steadily decaying signal [101,184,203]. Adsorption on the layer provides additional nonradiative pathways for the dissipation of the excitation energy, which is most likely lost as heat to the surroundings, reducing the fluorescence signal. Quenching of the signal by interaction with oxygen, or reaction of the solute with oxygen to produce new products with a diminished fluorescence yield, are other known mechanisms affecting the magnitude and stability of the fluorescence response.

The extent of fluorescence quenching often depends on the layer type and is usually more significant for silica gel than for chemically bonded layers. In some cases, the emission signal can be enhanced if the layer is impregnated with a viscous liquid before scanning. Common fluorescence-enhancing reagents include solutions of liquid paraffin, Triton X-100 and Fomblin oils [101,184,204]. The general mechanism of fluorescence enhancement is assumed to involve (partial) dissolution of the adsorbed analyte with an increase in emission resulting from the fraction of analyte transferred to the liquid phase where fluorescence quenching is less severe. In favorable cases, a signal enhancement of 10- to 200-fold is obtained. In those cases where fluorescence quenching is negligible, the application of a fluorescence-enhancing reagent rarely produces a significant increase in emission. Solvents of high viscosity are employed as fluorescence enhancing reagents to minimize zone broadening due to diffusion of the dissolved analyte during the measurement process.

Oxygen is a ubiquitous fluorescence-quenching reagent and is difficult to exclude from the layer surface during scanning densitometry. Flooding the scanning stage with nitrogen is possible. It is generally more practical, however, to impregnate the layer with an antioxidant, such as BHT (2,6-di-*tert*-butyl-4-methylphenol) before sample application, and to add antioxidant to the mobile phase [101,184,205]. Some adsorbed samples undergo photooxidation reactions, requiring the presence of an antioxidant and precautions to shield the sample from light. Fortunately, the above problems are not severe in most cases, and only those substance that are unusually susceptible to catalytic oxidation or oxygen quenching require special handling.

6.9.3 Slit-Scanning Densitometers

Instruments for scanning densitometry share many features in common, Figure 6.20 [8,9,11,104,140,184,204]. They usually allow measurements in the reflectance or transmission mode, by absorbance or fluorescence. Halogen or tungsten lamps are used for the visible range and deuterium lamps for the UV region. High-intensity mercury or xenon arc lamps are used for fluorescence measurements. In most cases, a motorized grating monochromators is used for wavelength selection and to record absorption spectra. For fluorescence measurements a filter, which transmits the emission wavelength envelope but attenuates the excitation wavelength, is placed between the detector and the plate. Photomultipliers or photodiodes are generally used for signal measurements. The plate is scanned at speeds up to about 10 cm/s by mounting it on a movable stage controlled by stepping motors. For lane scanning a fixed sample beam is shaped into a rectangular area on the plate surface through which the plate is transported. Some instruments have a turntable-type scanning stage for peripheral or radial scanning of circular and anticircular chromatograms. Alternatively, for point scanning the measuring beam is shaped into a spot or rectangle with dimensions much smaller than the sample zones. By moving the scanning stage in the x and y direction a zigzag or meander scan is initiated. Zigzag and meander scanning allow zones of any shape to be quantified. Distorted separations resulting from lateral migration of sample components can be corrected by track optimization in which the sample zones are integrated as if the slit had moved along an optimum track from peak maximum to peak maximum. Dual-wavelength measurements can be used to minimize matrix interference if suitable wavelengths can be found for the measurements. Ideally, two similar wavelengths are required at which sample absorption occurs at only one wavelength. Since absorption spectra tend to be broad this criterion is not easy to meet in practice.

A laboratory-built scanning densitometer using a fiber optic bundle for illumination of the layer and collection of reflected light (or fluorescence) in conjunction with a photodiode array detector affords some attractive features that conventional scanning instruments lack [186,206,207]. These include: the simultaneous recording of spectra from 198-612 nm with a spectral resolution of 0.8 nm; simultaneous detection at multiple wavelengths; a spatial resolution < 0.16 mm; and implementation of a wide

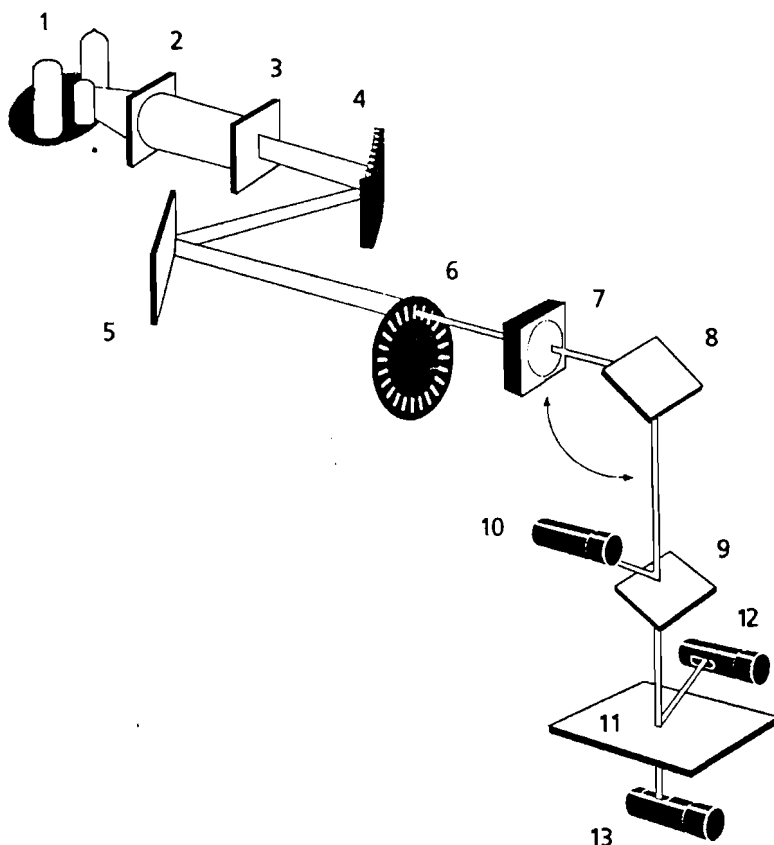


Figure 6.20. Optical arrangement for a slit-scanning densitometer (Camag scanner 3). Identification: 1 = lamp selector; 2 = entrance lens slit; 3 = monochromator entry slit; 4 = grating; 5 = mirror; 6 = slit aperture disk; 7 = lens system; 8 = mirror; 9 = beam splitter; 10 = reference photomultiplier; 11 = TLC plate; 12 = measuring photomultiplier; and 13 = photodiode for transmission measurements. (From ref. [8]; ©Marcel Dekker)

range of chemometric procedures for library searches, purity evaluation, resolution of overlapping zones, etc.

The principal sources of error in scanning densitometry are the reproducibility of sample application, the reproducibility of chromatographic conditions, the reproducibility of positioning the spot in the center of the measuring beam, and the reproducibility of the measurement [31,208,209]. The measurement error can be determined by repeatedly scanning a single lane of the thin-layer plate without changing any experimental variables between scans. It is composed of errors due to the optical measurement, electronic amplification, and the recording device. The measurement error is dependent on the signal-to-noise ratio, but for a properly adjusted instrument, typical relative standard deviation values fall into the range 0.2-0.7%. The positioning error is negligible if band

application or meander scanning is used and can be made very small in linear scanning of spots if good analytical practices are followed. The sample application error can be determined by applying standards to the layer and scanning the applied zones before development without changing any experimental variables between scans. The relative standard deviation for application errors using automated sample applicators is about 1.0 %. If the plate is then developed and rescanned without changing any of the experimental variables the chromatographic error can be estimated. This is quite often the most significant error and is only reduced by minimizing variations in the development process. The relative standard deviation from all errors in scanning densitometry can be maintained below 2-3 % for high performance layers.

6.9.4 Video Densitometers

For video densitometry optical scanning takes place electronically, using a computer with video digitizer, light source, monochromators and appropriate optics to illuminate the plate and focus the image onto a charged-coupled device (CCD) video camera [11,103,210-213]. The plate is evenly illuminated with monochromatic light and the reflected light focused as a scaled image of the plate directly onto the active element of the CCD camera. The CCD camera functions as a two-dimensional array of unit detectors (pixels). Photons colliding with an individual pixel result in the creation of a charge stored by an applied voltage. Accumulated charges on each pixel are then transported to a readout amplifier using sequential clocking of the applied voltage and the signal digitized for computer analysis. The captured images are initialized, stored, and transformed by the computer into chromatographic data. Background subtraction and thresholding are common data transformation processes. For background subtraction the accumulated images of a blank plate are subtracted from the analytical plate on a pixel-by-pixel basis. Thresholding is used to prevent the occurrence of negative values for the plate luminescence.

The main attractions of video densitometry for detection in thin-layer chromatography are fast data acquisition, absence of moving parts, simple instrument design, and compatibility with data analysis of two-dimensional chromatograms that are difficult to scan using conventional slit-scanning densitometers. However, given the limitation of today's technology, video densitometers cannot compete with mechanical scanners in terms of sensitivity, resolution, and available wavelength measuring range, but these instruments continue to improve all the time. They have proven popular in the development of field-portable instruments and as a replacement for photographic documentation of thin-layer separations. Modern instruments provide attractive options for searching and comparing sample images as well as integration of peak areas [214,215]. Most low-cost instruments are designed for operation in the visible region or UV region when layers containing a fluorescence indicator are used. Surprisingly good results were obtained with a conventional office flat bed scanner modified for scanning thin-layer plates by inclusion of a light source [216,217].

6.9.5 Miscellaneous Methods

Detection in thin-layer chromatography is dominated by optical methods using scanning densitometry with video densitometry as an increasingly popular alternative. Both approaches, however, yield similar information. Other, alternative methods, are useful because they provide complementary information, even if used less frequently. Identification methods based on mass spectrometry (section 9.2.2.5) and infrared and Raman spectroscopy (section 9.3.1.4) and fluorescence line narrowing spectroscopy are discussed elsewhere [11,218,219]. Flame ionization has been used for the detection of samples lacking a chromophore for optical detection. The separation is performed on specially prepared thin, quartz rods with a surface coating of adsorbent attached to the rod by sintering [80,81,202,220]. The rods are developed in the normal way, usually held in a support frame that also serves as the scan stage after the rods have been removed from the developing chamber and dried. Several rods can be held in the support frame and automatically scanned in order. The rods are moved at a controlled speed through a hydrogen flame and the signal processed in a similar manner to the flame ionization detector used in gas chromatography. The linear working range of the detector is about 3-30 μg for most substances and the response nearly universal. Other detector options include the thermionic ionization and flame photometric detectors for element-selective detection.

Biologically active compounds separated by thin-layer chromatography are easily revealed by bioautographic detection (biomonitoring) [11]. The thin-layer plate is simply dipped or sprayed with an enzyme such as cholinesterase and a substrate to determine inhibition by color differentiation [221,222]. Alternatively, the plate can be dipped into a suspension of luminescent bacteria with toxic substances revealed as dark zones resulting from reduced bioluminescence [223-225]. Antibacterial compounds can be detected by contacting the layer face-to-face with an inoculated agar plate. The separated zones are transferred to the agar gel by diffusion and inhibition zones visualized by use of suitable stains and (mainly) dehydrogenase-activity-detecting reagents [11,226]. Thin-layer chromatography with biomonitoring is a useful technique for toxicity-directed wastewater analysis [224]. This approach eliminates the time consuming work-up of fractions for biological testing and substantially reduces the expenditure for testing itself. To avoid the time consuming isolation of already known active compounds, a dereplication step (recognizing the already known active compounds) is required for the isolation of novel biologically active agents in screening plant and animal sources. Thin-layer chromatography with biomonitoring is well suited to this purpose, and allows unique compounds to be identified for isolation from complex extracts. Thin-layer chromatography with biomonitoring was also shown to be useful for high throughput screening of combinatorial libraries [226].

Modern imaging detectors for radiolabeled compounds employ a range of different technologies for the position sensitive detection of β - and γ -emitting isotopes of (mainly) ^3H , ^{14}C , ^{32}P and ^{125}I [227-233]. These instruments are only likely to be found in laboratories that handle radiolabeled compounds on a routine basis and have the necessary safety and training protocols in place. For detection multiwire proportional

counters, microchannel array detectors or phosphor imaging analyzers are used. Most detectors provide a spatial resolution of < 1 mm and are able to detect zones of radioactivity on a plate containing less than 10 dpm in a relatively short exposure time. These computer-based systems offer a variety of methods for data analysis and display.

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Chapter 7

Supercritical Fluid Chromatography

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7.1 INTRODUCTION

Supercritical fluid chromatography (SFC) has been seeking a permanent home in analytical laboratories since its inception in the early 1960s [1-10]. During this time it has enjoyed peaks of passing popularity championed as an extension of gas chromatography or a replacement for high-pressure liquid chromatography. In reality it is neither of these and a bit of both. Supercritical fluid chromatography is able to extend the molecular weight separating range of gas chromatography at the expense of some loss in efficiency and longer separation times. In the late 1980s supercritical fluid chromatography was reborn largely as an open tubular column technique using column and detector technology imported from gas chromatography and mobile phase delivery and injection systems from liquid chromatography [1-5]. The compromises were generally unsatisfactory. The small optimal column internal diameters combined with their low flow rates impeded instrument development and the reliance on carbon dioxide as virtually the only convenient mobile phase for use with flame-based detectors restricted applications to compounds with adequate solubility in carbon dioxide, a relatively non-polar solvating medium. Open tubular column supercritical fluid chromatography found a niche for itself in the separation of (generally) thermally stable compounds of low polarity (such as hydrocarbon-based and siloxane-based polymers, petroleum products, fats and oils, etc). These compounds were difficult to separate by gas chromatography because extreme temperatures were required to obtain adequate volatility. Simultaneously it acquired a reputation for being unsuitable for the separation of polar compounds because of poor peak shapes and, in some cases low mass recovery. Perhaps the main failing at this time was that excessive exuberance had raised expectations of the general capability of open tubular column supercritical fluid chromatography too high and using open tubular columns of the desired dimensions with available instrumentation was too problematic for entry into quality control laboratories. Contemporary use of open tubular column supercritical fluid chromatography has continued to decline during the 1990s with little expectations of an imminent resurgence of interest outside its niche application areas.

Supercritical fluid chromatography was reincarnated in the 1990s as a packed column technique, primarily as a replacement for normal-phase liquid chromatography, and mostly for the separation of polar compounds [6-10]. Uptake has been slow but steady during the last decade, faced with reservations based on poor experiences with open tubular column supercritical fluid chromatography and confusion over the new and contrary direction, seemingly against the accepted view of the limitations of supercritical fluid chromatography. This change in ideology was fostered by the movement to instrumentation more like that used in liquid chromatography, improved column packing materials, the general use of binary mobile phases containing additives to control undesirable column interactions, and the switch to spectrophotometric detectors with high-pressure cells for routine applications. Most modern instruments use reciprocating pumps specifically designed for compressible fluids and provide for independent pressure, flow and temperature control. They also allow accurate

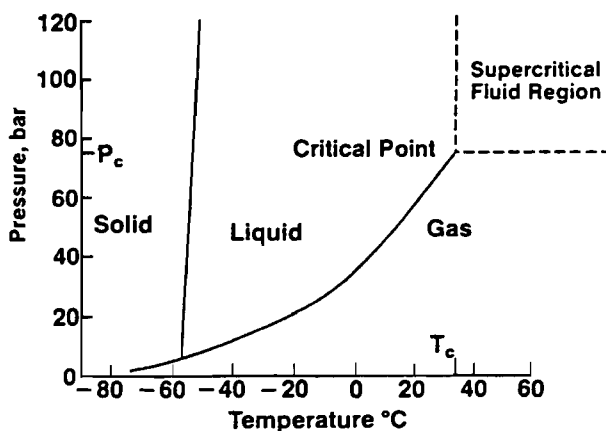


Figure 7.1. Phase diagram for carbon dioxide

mixing of compressible fluids and liquids, which is essential now that composition variations rather than density variations are the most widely used approach for method development. Fluid-solvent mixtures are far less compressible than neat supercritical fluids ameliorating some of the problems of efficiency loss and mobile phase velocity and retention heterogeneity for columns containing small particles and/or of longer lengths.

A supercritical fluid is simply an element or compound at a temperature above its critical temperature that is simultaneously compressed to a pressure exceeding its critical pressure, Figure 7.1. The supercritical fluid region is not a separate state of matter but simply an extension of the gas and liquid phase regions to a temperature and pressure domain where phase separation is no longer possible. The transition of physical properties from the gas or liquid phase to the supercritical fluid region is smooth and not marked by the dramatic changes that generally accompany transitions at phase boundaries, such as the melting of a solid or boiling of a liquid. The properties of supercritical fluids (e.g. density, viscosity, diffusivity, etc.) are continuously variable by changing the temperature and/or pressure above the critical point. The fluid may exhibit gas-like and liquid-like properties at the extremes of its range represented by the dotted lines in Figure 7.1 and some combination of these properties, at other locations that depend on the temperature and pressure coordinates. Table 7.1 provides some representative data for the density of carbon dioxide at different temperatures above the critical temperature at 72 atmospheres (close to the critical pressure) and 400 atmospheres (close to the highest pressure normally used in supercritical fluid chromatography). For carbon dioxide virtually the whole density range from about 0.1-1.0 g / ml is easily accessible representing a wide range of fluid properties.

Typical properties of a gas (helium) under gas chromatographic conditions, a liquid (water) under liquid chromatographic conditions, and carbon dioxide under low and high density conditions, as might be used in supercritical fluid chromatography, are

Table 7.1

Change in density of supercritical fluid carbon dioxide with pressure and temperature

Temperature (°C)	Pressure (atm)	Density (g/ml)
40	72	0.22
	400	0.96
60	72	0.17
	400	0.90
80	72	0.14
	400	0.82
100	72	0.13
	400	0.76
120	72	0.12
	400	0.70
140	72	0.11
	400	0.64

Table 7.2

Representative properties of typical chromatographic mobile phases

Mobile phase	Temperature (°C)	Pressure (atm)	Density (g/ml)	Diffusivity (cm ² /s)	Viscosity (cP)
Helium	200	1.5	2×10^{-4}	0.1-1	0.02
Carbon dioxide					
low density	100	80	0.15	10^{-3}	0.02
high density	35	200	0.8	10^{-4}	0.1
Water	20		1.0	10^{-5}	1.0

summarized in Table 7.2 [11]. The capacity of a mobile phase for intermolecular interactions is related to its density. Supercritical fluids have densities generally between those of gases and liquids but closer to those of liquids. They are expected to have higher solubilizing power than gases but are not as effective solvents as liquids. Unlike liquids, the density and solvating power of a supercritical fluid can be varied at a constant temperature by changing its pressure. Under normal conditions liquids are virtually incompressible and the external pressure is irrelevant to their solvent properties.

Diffusion coefficients in supercritical fluids are intermediate between those of gases and liquids but generally closer to those of liquids. They may be an order of magnitude greater than in liquids, however, which has important implications for chromatography affecting separation times, column characteristics and instrument design. Typical minimum plate heights for packed column supercritical fluid chromatography and liquid chromatography are similar. The most important difference is that the minimum plate height for supercritical fluid chromatography is achieved at linear velocities 5 to 10 times higher than for liquid chromatography, and separation times are reduced. Also, the resistance to mass transfer term in supercritical fluid chromatography is not as large as it is for liquids, which allows a further increase in the separation speed without a significant decrease in efficiency. It should always be possible to obtain faster separations by supercritical fluid chromatography than by liquid chromatography but

these separation speeds do not approach those of gas chromatography, even for low-density supercritical fluids.

The rate of radial diffusion controls the dimensions of open tubular columns when reasonable efficiency per unit column length is required. Open tubular columns need only have internal diameters of about 0.1-0.5 mm in gas chromatography to obtain reasonable efficiency, conditions that are easily met. For equivalent efficiency in liquid chromatography column internal diameters should be less than about 0.01 mm. These columns are neither easy to prepare nor simple to operate, and are rarely used in practice. The more favorable diffusion properties of supercritical fluids allow larger diameter open tubular columns to be used, about 0.05-0.10 mm, with relaxed instrument constraints compared to liquid chromatography. Thus, both packed and open tubular columns are used in supercritical fluid chromatography, where each type has distinct advantages, while for liquid chromatography packed columns are the only practical alternative. Since the solute diffusion coefficients vary strongly with fluid density, open tubular columns of typical dimensions will provide more favorable separation properties at low mobile phase densities. At high densities the column dimensions required for optimum performance approach those of liquid chromatography.

The viscosities of supercritical fluids are intermediate between those of gases and liquids, but are closer to those of gases. For a fixed column pressure drop longer columns or higher flow rates are possible in supercritical fluid chromatography compared with liquid chromatography. Supercritical fluids, however, are highly compressible and a large column pressure drop has the effect of decreasing the density along the length of the column and increasing the local mobile phase velocity leading to additional band broadening. For the same column pressure drop, the greater diffusivity of gases means that supercritical fluid chromatography cannot approach gas chromatography either in the total plate number that can be achieved or separation speed.

7.2 MOBILE PHASES

The properties desired of a supercritical fluid for use in chromatography are low critical constants, low chemical reactivity, low toxicity and flammability, availability in a high purity grade at a reasonable cost, and compatibility with common gas and condensed phase detectors. Only carbon dioxide meets these requirements for the most part. The critical properties of some other supercritical fluids are summarized in Table 7.3 [3,12]. Nitrous oxide has similar solvent strength to carbon dioxide but its strong oxidizing properties are a hazard [13]. Sulfur hexafluoride is a weaker eluent than carbon dioxide and difficult to obtain in adequate purity. Xenon has low solvent strength, is expensive, but has found some applications when combined with FTIR detection due to its favorable spectral transparency extending from the vacuum UV to the NMR region [14]. Low molecular mass n-alkanes are weak solvents, have relatively high critical constants, and cannot be used with flame-based detectors [3,15]. These fluids are flammable and require extra care when used.

Table 7.3

Physical properties of some supercritical fluids

Fluid	Critical Parameters			Density at 400 atm (g/ml)
	Temperature (°C)	Pressure (atm)	Density (g/ml)	
Carbon dioxide	31.3	72.9	0.47	0.96
Nitrous oxide	36.5	72.5	0.45	0.94
Sulfur hexafluoride	45.5	37.1	0.74	1.61
Xenon	16.6	58.4	1.10	2.30
Butane	152.0	37.5	0.23	0.50
Pentane	196.6	33.3	0.23	0.51
Dichlorodifluoromethane	111.8	40.7	0.56	1.12
Trifluoromethane	25.9	46.9	0.52	1.15
1,1,1,2-Tetrafluoroethane	101.2	40.2	*	
Ammonia	132.5	112.5	0.24	0.40
Water	374.4	226.8	0.34	
Methanol	240.5	78.9	0.27	
Acetonitrile	274.7	47.7	0.24	

* Density = 1.147 at 75°C and 200 bar and 0.985 at 125°C and 200 bar.

All the above fluids are weak mobile phases [16,17]. Carbon dioxide has similar solvent properties to liquid hydrocarbons with the principal intermolecular interactions being dispersion and induction, since carbon dioxide has no dipole moment of its own. Polar substances capable of a wider range of intermolecular interactions tend to have (excessively) high critical constants incompatible with the stable operation of contemporary columns and instruments (see Table 7.3). Of the potentially useful polar fluids the chlorofluorocarbons and hydrofluorocarbons have favorable critical constants and modest dipole moments, but availability, cost and environmental concerns have limited their use [18,19]. The dipolar and hydrogen-bond basic hydrofluorocarbon refrigerant 1,1,1,2-tetrafluoroethane (HFC-134a) has a reasonable potential for future use [20]. Ammonia is a strong solvent but is too aggressive and toxic for practical use [21,22]. It dissolves silica-based materials and corrodes instrument components.

Carbon dioxide is available in a number of grades, including a purified supercritical fluid chromatography grade, and is sold in pressurized cylinders in which the bulk of the carbon dioxide is in the liquid state. Cylinders with a helium headspace are available to assist in dispensing liquid carbon dioxide without having to cool pump heads, etc. These cylinders are unsuitable for use in supercritical fluid chromatography when reproducible retention is important [23-25]. Under typical conditions the concentration of helium dissolved in the liquid carbon dioxide varies from about 0 to 5 mole percent. The lower elution strength of carbon dioxide-helium mixtures compared with carbon dioxide alone results from the significant reduction in the density of the mixed mobile phase and variation in retention is related to changes in the composition of the dispensed carbon dioxide-helium mixture as the cylinder contents are consumed. Cylinder lubricants, a mixture of chlorotrifluoroethylene oligomers, have been identified as trace contaminants in both high and low purity grades of carbon dioxide [26]. Primary

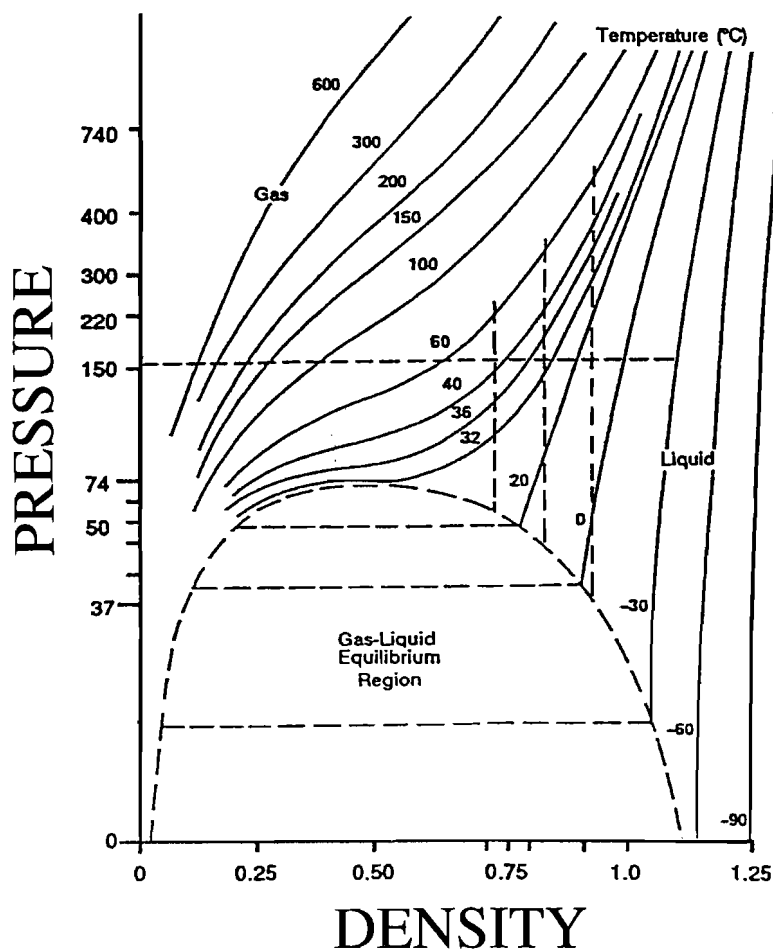


Figure 7.2. Isotherm diagram for carbon dioxide. Broken lines indicate crossing of isotherms at either constant pressure or density.

and secondary amines with a $pK_b > 9$ might react with carbon dioxide to form urea and carbamate derivatives [27], although there are few reports of problems in the separation of aromatic amines and N-heterocyclic compounds.

The solubilizing power of a supercritical fluid depends on its density and capacity for specific intermolecular interactions. The density is related to the experimental parameters of pressure and temperature in a non-linear manner, Figure 7.2, described by an equation of state. Close to the critical point the density changes markedly in a sigmoidal manner with small changes in pressure. At temperatures further removed from the critical point the isobars are flatter and the change in density with pressure is

approximately linear. Two competing effects govern solubility in a supercritical fluid [28-30]. As temperature increases the vapor pressure of the solute tends to increase its solubility. At the same time increasing temperature tends to lower the fluid density and to decrease its solvating power. At higher pressures the fluid density changes less with temperature and solute solubility depends largely on vapor pressure. At lower pressures solubility is likely to change markedly with temperature and decreases significantly with increasing temperature reflecting large changes in fluid density. These results reveal the importance of controlling density in supercritical fluid chromatography and the rather complex relationship between pressure and temperature that control density. Increasing the fluid density at constant temperature will normally increase solubility providing the appropriate conditions for samples to migrate through the column in supercritical fluid chromatography. A certain minimum density is required to elute a sample at a given temperature, and if the pressure cannot be adjusted to obtain this density, then the sample will not be eluted [28,31]. At a constant density, increasing the temperature will usually reduce retention.

7.2.1 Mixed Mobile Phases

The general approach to enhance the range of selective mobile phases available for supercritical fluid chromatography is to use binary mixtures of a supercritical fluid, usually carbon dioxide, and a polar organic solvent [32-35]. The addition of an organic solvent alters the selectivity and elution strength of the mobile phase by increasing its polarity and density, and deactivates active sites on the stationary phase. This results in increased solubility for high molecular mass and polar compounds, improved peak shapes, and shorter separation times. Applications generally fall into two categories. Polar solvents of low solubility in carbon dioxide used with small bore columns and flame-based detectors and solvents that form single-phase mixtures with a reasonably high mole fraction of the modifier and generally used with packed columns and spectrophotometric detectors.

When flame ionization detection is employed water [10,36-39], formic acid [38,40,41] and formamide [41] are the most suitable modifiers for carbon dioxide. These modifiers are sparingly soluble with a maximum concentration of about 0.3% (v/v) in liquid carbon dioxide at room temperature. Saturated solutions at these mixing ratios are easy to prepare but other concentrations are problematic [38,40]. For water a saturator column containing coarse silica coated with water and positioned between the pump and injector is satisfactory if of a limited lifetime [39,40]. A porous membrane loaded with water also provides a suitable reservoir for saturating liquid carbon dioxide with water [39]. Formic acid is often a more effective modifier than water due to its greater capacity to compete with polar solutes for hydrogen-bonding sites on the stationary phase, and perhaps, because of its capacity to suppress ionization of weak acids. The deactivation properties of formic acid are illustrated by the separation of organotin compounds using supercritical fluid carbon dioxide saturated with formic acid in Figure 7.3 [41,42]. In the absence of formic acid the separation was totally unsatisfactory with several compounds

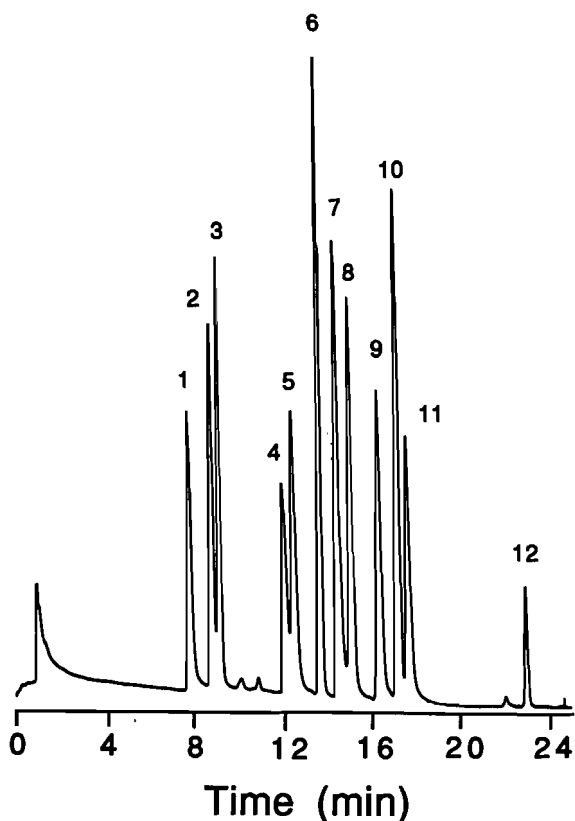


Figure 7.3. Separation of organotin compounds on a 10 cm x 1 mm I.D. column packed with Deltabond Methyl with supercritical fluid carbon dioxide saturated with formic acid as mobile phase. The separation was obtained at 60°C using pressure programming: 0.5 min hold at 90 atm. Then programmed at 4 atm / min to 150 atm where the program rate was increased to 10 atm / min to 300 atm. Peak identification: 1 = dibutyltin dichloride; 2 = tributyltin chloride; 3 = tetrabutyltin; 4 = diphenyltin dichloride; 5 = dicyclohexyltin dichloride; 6 = bis(tributyltin) oxide; 7 = triphenyltin chloride; 8 = tricyclohexyltin chloride; 9 = tetraphenyltin; 10 = tetracyclohexyltin; 11 = bis(triphenyltin) oxide; and 12 = hexakis(2-methyl-2-phenylpropyl) distannoxane. (From ref. [42]; ©Springer-Verlag)

completely adsorbed by the stationary phase. Formic acid has a rather poorly defined composition, is unstable and difficult to purify, and may accelerate the deterioration of some instrument components [38,43]. Formamide is a weaker hydrogen-bond acid and a significantly stronger hydrogen-bond base than formic acid [41]. It is particularly useful for eluting tertiary amines (but not primary or secondary amines) from silica-based chemically bonded stationary phases. The flame-ionization detector response to formamide is greater than that for formic acid resulting in a four-fold higher baseline rise during density programming.

At the low mixing ratios for water, formic acid and formamide in carbon dioxide the modifier is unlikely to have a significant influence on the solubility of the sample in the mobile phase, and probably acts primarily by masking interactions between the sample and accessible silanol groups of the stationary phase. If these interactions make a significant contribution to retention of the sample then in the presence of a suitable modifier a substantial reduction in retention will occur, but more typically, smaller changes in retention with improvements in peak symmetry are observed. When the same modifiers are used with relatively inert fused silica open tubular columns at the same concentration, the change in chromatographic behavior is often very small compared to the unmodified mobile phase, indicating that the modifier behaves primarily as a masking agent for stationary phase silanol groups. In which case, the above modifiers should be considered as a means of extending the deactivation of well-deactivated, silica-based stationary phases.

The use of non-flame based detectors, an increasingly popular option in supercritical fluid chromatography, allows greater flexibility in the selection and composition variation of mixed mobile phases. The minimum temperature and pressure required to ensure that a binary mobile phase remains in the supercritical fluid region can be obtained from phase diagrams, if available, or estimated using various numerical techniques based on an appropriate equation of state and interaction parameters, or determined experimentally [32,33]. Most studies have been performed using high-pressure view cells and either visual observations or light scattering detection. A simple solvent peak-shape method, employing flow injection analysis using chromatographic equipment, was instrumental in providing a comprehensive collection of critical-mixture curves for all common binary mixtures of organic solvents in carbon dioxide of likely interest for supercritical fluid chromatography [33,44,45]. The organic modifiers generally used in SFC exhibit either type I or type II phase behavior depicted in Figure 7.4 (a comprehensive account of phase behavior would consider all six possible characteristic phase diagrams [32]). Type I mixtures are formed when the two solvents as liquids are miscible and type II mixtures when the two solvents as liquids are immiscible but become miscible near the critical region. Increasing the mole fraction of a polar organic solvent in carbon dioxide usually causes a continuous increase in the critical temperature while the critical pressure at first rises to a maximum and then falls back at higher mole fractions of modifier. Because of these constraints the portion of the phase diagram of interest for supercritical fluid chromatography is usually 0-20 mole percent of organic solvent, 0-200°C temperature and 40-400 atm pressure. Mixed phases with high critical constants are problematic in use and may decompose either solutes or stationary phases. The dotted line in Figure 7.4, representing the critical mixture curve spanning the entire composition range of critical temperatures and pressures, indicates the conditions where a single component supercritical phase exists and the two phase liquid and vapor region. Actually, for separations it is not important whether the mixture is a supercritical or sub-critical mixture provided that the mobile phase is a single phase. Phase separation in supercritical fluid chromatography can lead to broadened, split and irregular peaks, excessive baseline noise, and irreproducible

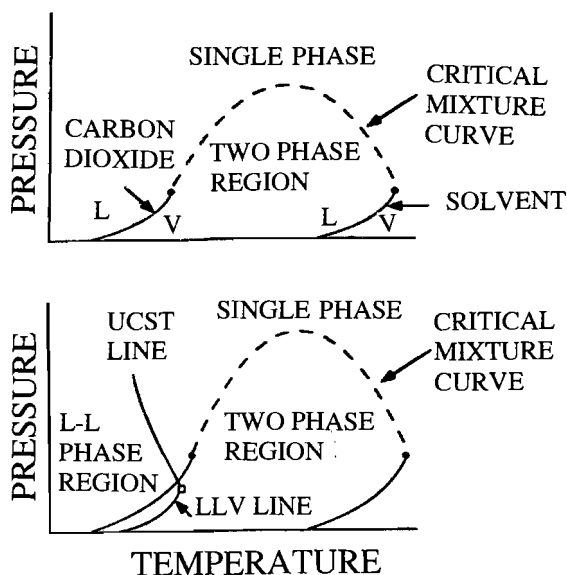


Figure 7.4. Phase diagrams for type I (top) and type II (bottom) binary mixtures with carbon dioxide as one component (L = liquid and v = vapor). The UCST line indicates the temperature at which the two immiscible liquids merge to form a single liquid phase. The critical mixture curve is the locus of critical mixture points spanning the entire composition range. (From ref. [44]; ©American Chemical Society).

retention. In general terms, only carbon dioxide with methanol or acetonitrile as modifiers has reached anything like routine practice. Binary mobile phases often provide inadequate retention on open tubular columns and their use is less common than with packed columns. Also the range of densities possible with mixed mobile phases is considerably reduced compared with pure carbon dioxide and density changes are far less powerful as a method of retention optimization. Composition and temperature variation tends to be the dominant optimization strategy when using mixed mobile phases. The adsorption of mobile phase components by the stationary phase plays an important role in controlling retention and efficiency (section 7.4).

7.2.2 Additives

Polar solvents are effective for controlling the elution strength of the mobile phase and adjusting the capacity of the mobile and stationary phases for selective solute interactions, but are incapable of solving all problems that limited the application of packed columns to the separation of polar compounds. The use of additives, very polar substances added to the mobile phase at low concentration, some of which are expected to improve peak shapes by masking or reacting with active sites, are credited with opening up the application range of packed column supercritical fluid chromatography to include compounds such as fatty acids, glycerides, amides, sulfonamides, saccharides, steroids,

and complex pharmaceutical and industrial compounds containing carboxylic acid or amine functional groups [6-8,46,47]. These, now routine applications, were thought to be unlikely candidates for separation by supercritical fluid chromatography less than a decade ago. Most additives of interest for suppressing analyte interactions with silanol groups are polar substances with limited solubility in carbon dioxide. When solvent modified fluids are used this disadvantage disappears and the number of useful additives is much larger. The additive is usually added to the solvent modifier and the modifier with additive pumped as a single solvent. It is quite straightforward to obtain stable chromatographic systems but since the additive is adsorbed by the stationary phase changing additives or switching to non-additive conditions may require lengthy equilibration times. Also, since the additive concentration is easily varied in solvent modified fluids, additives can be used to adjust the selectivity of both the mobile and stationary phases, if desirable, in addition to their basic role of masking sorbent active sites. Acid additives, such as trifluoroacetic acid, citric acid, and trifluoromethylsulfonic acid are used to improve the chromatographic properties of acidic compounds like carboxylic acids and phenols [47-50]. Basic additives, such as propylamine, triethylamine or triethanolamine are used to improve the chromatographic properties of N-heterocyclic compounds and anilines [51-53].

There are only general guidelines for the selection of an additive. Stronger acids (bases) make the best additives for suppressing tailing and improving peak shapes of acidic (basic) compounds. Additives seem to be most effective on polar stationary phases, perhaps due to greater adsorption by the stationary phase.

7.3 STATIONARY PHASES

Nearly all practical applications of SFC are performed using packed columns designed for high-pressure liquid chromatography or fused-silica open tubular columns with immobilized phases similar to those used in gas chromatography. In the latter case, columns of smaller internal diameter, 25-100 μm , shorter lengths (generally less than 20 m with 1-10 m being common), and more firmly crosslinked stationary phases are used by comparison with standard columns for gas chromatography.

Totally porous and pellicular silica packings are used in adsorption chromatography for the separation of (usually) non-polar and moderately polar compounds [54-56]. The eluotropic strength of common supercritical fluids is not very high which prevents the elution of polar solutes in a reasonable time and/or with an acceptable peak shape. Mixed mobile phases containing a polar solvent such as methanol, extend the polarity range of compounds that can be eluted but often at the expense of selectivity. Consequently, silica-based, chemically bonded sorbents are the most widely used for the separation of polar and high molecular mass compounds.

Chemically bonded phases can be prepared in a number of ways (section 4.2.2) but invariably contain unreacted and poorly shielded silanol groups, even after the most

exhaustive chemical treatment. These residual silanol groups can cause tailing and adsorption of polar solutes, particularly organic bases and solutes with hydrogen bonding functional groups. They are typically used with mixed mobile phases containing polar organic solvents and possibly additives, as discussed in the previous section. For supercritical fluid carbon dioxide alone or with low concentrations of organic modifiers encapsulated phases (section 4.2.3) generally exhibit the lowest silanol activity [10,34,57-60]. Sorbents of moderate surface area based on silica microparticles with 30 nm and 50 nm pore sizes are commercially available with different immobilized ligands, including methyl, octyl, octadecyl, phenyl and cyanoalkyl with a poly(siloxane) backbone, and a poly(ethylene glycol) phase. Poly(siloxane) prepolymers containing polar substituent groups (e.g., alkanediol, cyclodextrin, cyanobiphenyl, liquid crystal, etc.) allow a wide range of polar chemically bonded phases to be synthesized, but most of these are described in the research literature only [61-66]. Although encapsulation shields silanol groups from the sample, it is unable to prevent all interactions with the sample, because the immobilized film may not cover the whole surface leaving some of the substrate groups exposed and/or because samples soluble in the polymer film must have access to the substrate by diffusion through the film.

Sorbents of a different structure could potentially circumvent some of the problems associated with the adsorptive activity of silica-based materials. Macroporous polymeric beads, developed for reversed-phase liquid chromatography (section 4.2.4), are chemically inert but have gained little favor in supercritical fluid chromatography. Early versions of these materials deteriorated rapidly under typical supercritical fluid chromatographic conditions. The constant swelling and shrinking of the beads in response to changes in the absorption of mobile phase components resulted in mechanical disintegration. Highly crosslinked poly(divinylbenzene) and poly(styrene-divinylbenzene) polymers with adequate mechanical stability are now available [67,68]. With carbon dioxide as the mobile phase, retention is high compared with chemically bonded phases, and the efficiency of well-retained compounds is often less than that for earlier eluting peaks. This has tended to limit applications to the separation of low molecular mass compounds found difficult to elute from silica-based sorbents without the use of modifiers [69,70]. The elution of higher molecular mass samples in an acceptable separation time was demonstrated using mobile phases containing modifiers [71]. It remains unclear, however, whether compounds, with a molecular mass over 1000, can be separated on these columns. One contributing factor to the strong retention may be the sorption of mobile phase components by the sorbent resulting in an unfavorable phase ratio for fast separations and the elution of high molecular mass compounds.

Porous graphitic carbon (section 4.2.5) is an inert but highly retentive sorbent under supercritical fluid chromatography conditions. Supercritical carbon dioxide is a weak eluent for porous graphitic carbon and even compounds such as naphthalene are difficult to elute in a reasonable time [72]. Low molecular mass polar compounds generally have poor peak shapes, but in this case most likely due to limited solubility in the mobile phase rather than undesirable interactions with active sites on the stationary phase. The flat surface of porous graphitic carbon leads to preferential adsorption of

planar compounds and useful selectivity for the separation of stereoisomers. The use of modifiers considerably extends the molecular mass and polarity range of compounds that can be eluted by carbon dioxide from porous graphitic carbon [73,74]. The most effective modifiers are dipolar and higher mass solvents than the popular modifiers used with silica-based sorbents, but even so, the possible application range still seems limited to the separation of low molecular mass compounds.

Silver-loaded sorbents are used for the separation of compounds according to the number, position and configuration of double bonds through selective complexation with silver ions [75]. Applications of silver-loaded sorbents in SFC have been limited so far to the separation of lipids using silver-loaded, cation-exchange [76-79] or silver-complexed chemically bonded sorbents [79-81]. All sorbents provide a good separation of fatty acid methyl esters according to their degree of unsaturation but the silver-loaded, cation-exchange sorbents require the use of modifiers restricting the choice of detectors to spectroscopic or evaporative light scattering detectors. The silver-loaded, cation-exchange sorbents are suitable for the separation of triacylglycerols from seed oils as shown in Figure 7.5 [78] and in general provide different selectivity to separations by silver-complexation, normal-phase liquid chromatography.

7.4 KINETIC OPTIMIZATION

Theoretical approaches to column design in supercritical fluid chromatography are not as straightforward, or as well developed, as models used in gas and liquid chromatography. Also, many studies in this area are contradictory, in large part because simple models fail to account for changes in system characteristics associated with different operating domains. The mobile phase in supercritical fluid chromatography is compressible and non-ideal resulting in additional sources of band broadening that depends on the fluid density and its variation along the column [82-88]. The fluid density gradient creates two other gradients: a mobile phase velocity gradient and a solute retention factor gradient. Expansion of the mobile phase towards the column outlet increases the mobile phase velocity and reduces the mobile phase elution strength. The column pressure (density) drop is small for open tubular columns but is more significant for packed columns. For modified mobile phases density gradients are less important because of their higher average fluid densities, lower compressibility and smaller variation in elution strength with changes in density. Small changes in experimental conditions close to the critical point show the most dramatic variation in column performance and this region should be avoided for general applications. It has been suggested that the loss of efficiency associated with a large pressure drop is the result of the formation of radial temperature gradients in uninsulated columns due to expansion and cooling of the mobile phase [89]. This results in a density and viscosity gradient from the center of the column towards the column wall.

Mobile phase modifications of stationary phase properties, that are also expected to vary with density, are a further complication [90-96]. The supercritical fluid

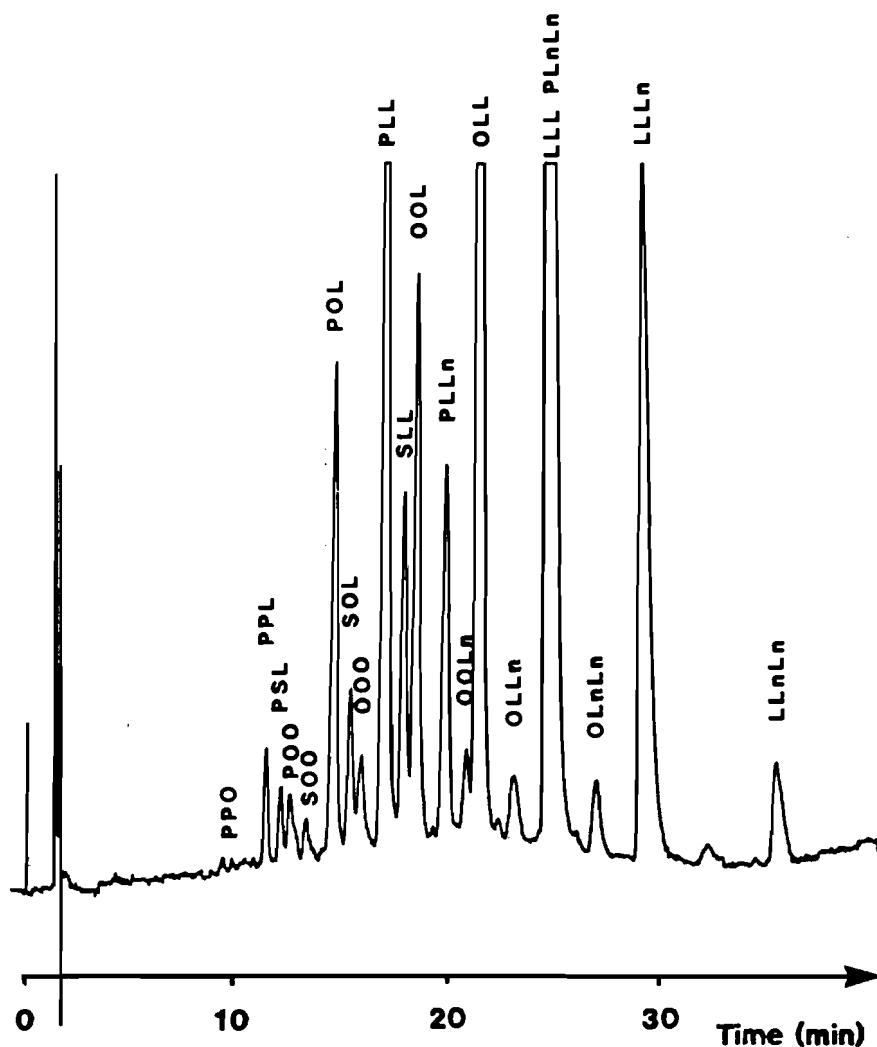


Figure 7.5. Separation of triacylglycerols in soybean oil on a silica-based, silver-loaded cation-exchange column (29 cm x 0.25 mm I.D.) with carbon dioxide-acetonitrile-isopropanol (2.8:6.5:0.7) as mobile phase. Start 115°C and 260 atm; after 2 min programmed at -1°C/min to 75°C and 1 atm/min to 300 atm. (From ref. [77]; ©Elsevier).

and modifiers will adsorb onto and/or absorb into solid and polymeric stationary phases, altering both the chemical and physical properties of the stationary phase. The absorption of mobile phase components causes the stationary phase to increase in volume and adsorption the creation of a film of stagnant liquid at the stationary phase surface. The adsorbed film is expected to have liquid-like densities more or

less independent of the mobile phase density. Diffusion in the adsorbed film can be slow relative to the lower density mobile phase. Slow diffusion in the adsorbed film and swollen stationary phase results in a significant increase in the mass transfer contribution to band broadening. With neat carbon dioxide multi-layer adsorption can result in a thick film when the temperature and density are both relatively low. The adsorption decreases with increasing temperature and is probably of little importance at temperatures greater than 100°C. Open tubular columns are generally operated in a temperature regime where adsorption of mobile phase is not of major importance, but this is not true for packed columns, particularly when mixed mobile phases are used.

Pressure, temperature and density relationships for supercritical fluids are normally described by a multi-parameter equation of state, which further complicates any mathematical treatment of the band broadening process [82,97]. It is generally assumed in formulating most models that the mobile phase density varies linearly with column length while more exact calculations indicate that this is not the case. Based on the numerical solution for the heat balance equation for packed columns operated under typical supercritical fluid chromatographic conditions the density profile along the column is either linear or slightly convex [98]. In most studies, instrumental contributions to band broadening and band broadening caused by limited solubility of the sample in the mobile phase were not taken into account, contributing to the inconsistent plate height values for similar conditions found in the literature.

7.4.1 Open Tubular Columns

The general approach for kinetic optimization of open tubular columns has been to adopt the familiar Golay equation (section 1.5.2) and to assume that the mobile phase can be approximated by an incompressible fluid with ideal gas properties [93,99-104]. Circumstances that are approximate at best, but serve adequately to demonstrate some of the fundamental characteristics of open tubular columns operated at low fluid densities. The column plate height equation can be written in the form given by Eq. (7.1)

$$H = [2D_M / u] + [f_g(k)d_C^2 u / D_M] + [f_s(k)d_f^2 u / D_S] \quad (7.1)$$

where H is the column plate height, u the average mobile phase velocity, D_M the diffusion coefficient of the solute in the mobile phase, D_S the diffusion coefficient of the solute in the stationary phase, d_C the column internal diameter, d_f the stationary phase film thickness, and $f_g(k)$ and $f_s(k)$ are functions of the retention factor explained elsewhere (section 1.4). Inserting appropriate values for the above variables into Eq. (7.1) allows a numerical comparison of different column types to be made, Table 7.4 [100-102]. The separation time and efficiency is strongly dependent on the column internal diameter. The optimum mobile phase velocity is inversely proportional to the column internal diameter, and for the same diameter, decreases as the retention factor increases. The column efficiency is highest at the optimum linear velocity but the separation times may be too long for practical applications. A linear velocity ten

Table 7.4

Influence of column internal diameter on efficiency and separation time for open tubular columns in supercritical fluid chromatography

$D_M = 2 \times 10^{-4} \text{ cm}^2/\text{s}$, $D_S = 1 \times 10^{-6} \text{ cm}^2/\text{s}$, $P = 72 \text{ atm}$, $T = 40^\circ\text{C}$, density = 0.22 g/ml and $d_f = 0.25 \text{ }\mu\text{m}$.

Column diameter (μm)	Optimum plate height and velocity at two retention factors					
	k = 1			k = 5		
	H_{\min} (mm)	$10u_{\text{opt}}$	u_{opt} (cm/s)	H_{\min} (mm)	$10u_{\text{opt}}$	u_{opt} (cm/s)
25	0.016	0.080	0.505	0.021	0.107	0.376
50	0.031	0.156	0.259	0.042	0.213	0.190
75	0.046	0.234	0.174	0.063	0.318	0.126
100	0.061	0.309	0.130	0.084	0.425	0.095

times the optimum velocity is more common in practice, resulting in about a five-fold increase in the minimum plate height for the range of retention factor values indicated in Table 7.4. For fast separations with a high plate number, column internal diameters below 0.1 mm are required and much smaller diameters would be preferred. The optimum column internal diameter is controlled by the rate of radial mobile phase mass transfer and decreases with increasing density due to unfavorable changes in the solute mobile phase diffusion coefficient. As the diffusion coefficients become more liquid-like column dimensions for acceptable performance approach values similar to liquid chromatography and are not easily attained experimentally. For the favorable case of low-density fluids it should be possible to obtain 2,000-9,000 plates per meter for retained solutes, Figure 7.6 [100,101]. Also, these values should not be strongly influenced by the stationary phase film thickness, at least up to about 1 μm , in contrast to observations made in gas chromatography. Thick films are of interest in open tubular column supercritical fluid chromatography because they allow an increase in sample loadability, which is of practical importance for trace analysis. For mobile phase velocities exceeding about 0.5 cm/s, which are required in practice for reasonable separation times, the contribution to the column plate height from longitudinal diffusion in the mobile phase is negligible compared to the contribution of mass transfer in the mobile and stationary phases.

7.4.2 Packed Columns

There is no exact form of the plate height equation for packed columns under supercritical fluid chromatographic conditions. It has generally been assumed that the Knox equation (section 1.5.3) developed for liquid chromatography can be used in the absence of a more definitive expression [1,2,98]. For high-density fluids and mixed mobile phases the Knox equation is probably adequate. At low densities with a high column pressure drop, predictions are expected to be less reliable. The variation in diffusion coefficients, linear velocity, viscosity and solute retention factors along the column for compressible fluids prevents any simple connection between model coefficients and column properties from being determined by curve fitting experimental plate height values as a function of the mobile phase velocity. An improved fit of

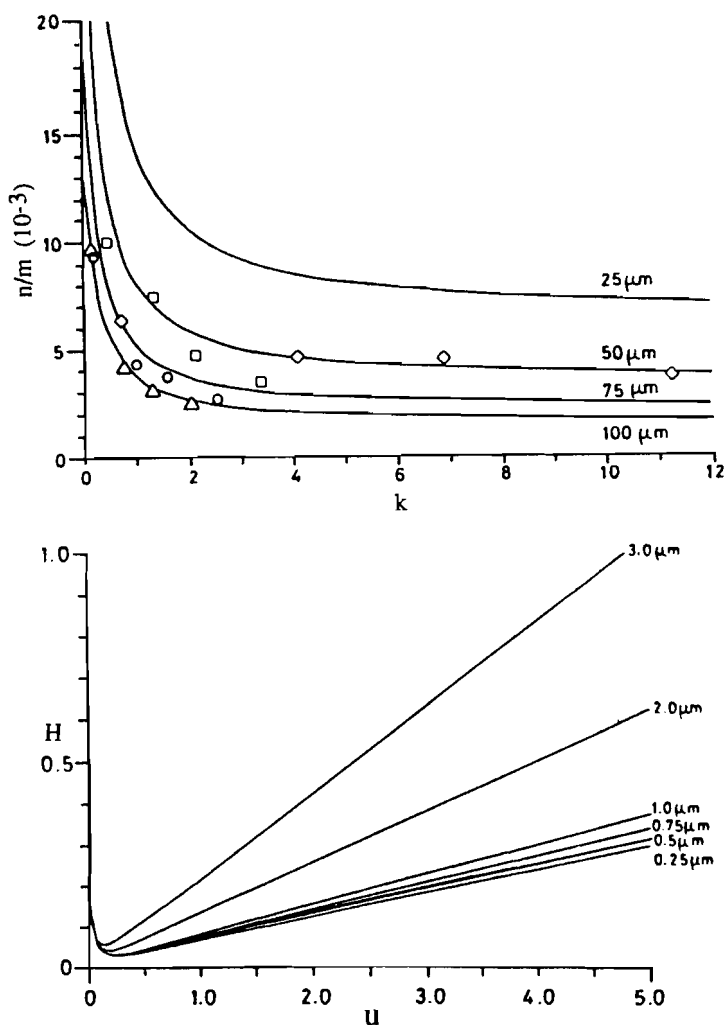


Figure 7.6. Affect of column internal diameter and stationary phase film thickness on efficiency for open tubular columns operated at $10 u_{\text{opt}}$ with low-density supercritical fluid carbon dioxide as the mobile phase. (From ref. [100 and 101]; ©Wiley-VCH)

experimental plate height values and column properties was claimed for a modified form of the Knox equation with a different dependency on the mobile phase velocity (calculation of the reduced velocity is problematic due to the variation of the mobile phase diffusion coefficients with changes in the column pressure drop) [105-107]

$$h = A + B' / u^{1.5} + C' u^{1.5} \quad (7.2)$$

where h is the reduced plate height, u the average mobile phase velocity and A , B' and C' are model coefficients related to column properties, but unlike liquid chromatography, the B' and C' coefficients are dependent on the column pressure drop. Interpretation in terms of column properties is not straightforward, however, and will not be discussed here.

In terms of separation speed there is qualitative agreement between theory and experimental results. For fast separations packed columns are preferred. These should be either short columns packed with small particles when only a limited plate number is required for the separation or long columns packed with larger particles (e.g. $d_p \approx 15 \mu\text{m}$) when a larger plate number is needed [105-108]. Open tubular columns of normal column diameters are capable of providing very large absolute plate numbers but produce relatively low plate numbers per unit time as well as low retention factors. Compared with packed columns, they are less useful for fast separations. In addition, packed columns have the advantage of a higher sample capacity providing a greater dynamic range for the determination of major and minor sample components.

A number of theoretical studies concluded that a pressure drop greater than 20 bar would cause a severe efficiency losses in packed column supercritical fluid chromatography [1,2,5,6,82]. With such small a pressure drop, the maximum column efficiency would be limited to approximately 20,000 plates. In practice packed columns providing over 100,000 plates per meter with a total plate number more than 200,000 and operated with a column pressure drop of about 160 bar have been demonstrated [86,109-112]. Coupled columns providing the highest total plate number were obtained with mixed mobile phases of low compressibility and demonstrate that the theoretical performance limit proposed for packed column supercritical fluid chromatography is too pessimistic.

7.5 RETENTION

Retention in supercritical fluid chromatography is a complex function of several variables and is not as easily rationalized as for gas and liquid chromatography. Retention in supercritical fluid chromatography is dependent upon pressure, density, temperature and composition of the mobile phase, the composition of the stationary phase, and in some cases, sample concentration. Many of these variables are interactive and do not change in a simple or predictable manner. The column phase ratio and composition of the stationary phase depend on the properties of the mobile phase and change significantly with alteration in mobile phase parameters. Certain general observations, however, can usually be applied [1,96,113-119]. Some largely empirical relationships that explain the influence of density and temperature on retention are summarized in Table 7.5 [82,94,119-121]. Changes in retention with temperature at a constant density are predictable from van't Hoff plots (section 1.4.4). The logarithm of the capacity factor is a linear function of the reciprocal of the column temperature, which persists down to sub-critical conditions. For separations at constant pressure

Table 7.5

Relationships characterizing the effect of density and temperature on retention

T = temperature (K), ρ = density, ρ_r = reduced density (ρ / ρ_c) and ρ_c = critical density.

Density varied at a constant temperature

$$\ln k = A + B\rho$$

$$\ln k = A + B\rho + C\rho^2$$

$$\ln k = A + B\ln\rho$$

$$\ln k = A - B\rho_r + C\rho_r^2$$

Temperature varied at a constant density

$$\ln k = A + B / T$$

Retention as a function of temperature and density

$$\ln k = A + (B / T) + C\rho + D(\rho / T) + E(\rho^2 / T)$$

$$\ln k = A + B\rho + C\rho^2 + (D / T) + E(\rho / T)$$

$$\ln k = A + (B / T) + C\rho + D(\rho / T) + E(\rho^2 / T)$$

$$\ln k = A + B\ln\rho + (C / T) + D(\ln\rho / T)$$

changing temperature may result in either an increase or decrease in retention for different solutes and possible changes in the elution order. Over a small density range the change in retention at a constant temperature is often linear but for a wider density range the relationship is generally non-linear. This inherent non-linearity is apparent in the form of the models summarized in Table 7.5. When organic modifiers are added to the mobile phase at constant pressure and temperature retention will either increase or decrease depending on whether the solutes are more or less soluble in the modifier compared to the supercritical fluid, assuming that the column activity level remains the same. The general change in retention with modifier concentration at a constant temperature is non-linear and can usually be described by a second order polynomial function of the mole fraction (or volume percent) of organic solvent.

The elucidation of a formal retention model for supercritical fluid chromatography is a complex problem. An early approach was based on a statistical thermodynamic treatment using a lattice-gas model to formulate a relationship for the solute partition coefficient [82,122,123]. This approach provided some useful insights into the retention process but is fairly involved mathematically and describes only segments of the experimental data for specific operating conditions. A regression model based on expanded solubility parameters to describe solute properties and a corresponding series of experimentally derived coefficients for the separation system properties was proposed [124]. The model successfully explained the large change in retention accompanying the addition of small amounts of methanol to supercritical fluid carbon dioxide but is restricted by the approximate method of calculating the solubility parameters and inconsistencies in their definition. The solvation parameter model (section 1.4.3) was used to study the retention mechanism in subcritical and supercritical fluid chromatography with mobile phases based on carbon dioxide in open tubular columns [125,126] and packed columns containing chemically bonded phases [127-129] and porous polymers [20,69,70]. In addition, limited information is available for

Table 7.6

Effect of pressure and temperature on the system constants of the solvation parameter model with carbon dioxide as the mobile phase

(5 m x 50 μ m open tubular column coated with the poly(dimethylsiloxane) stationary phase SB-Methyl-100).

Approximate pressure (atm)	Temperature ($^{\circ}$ C)	System constants				
		<i>l</i>	<i>r</i>	<i>s</i>	<i>a</i>	<i>b</i>
75.7	100	0.361	0.05	0.16	0.21	-0.06
89.4	100	0.330	0.06	0.15	0.21	-0.06
103.2	100	0.299	0.08	0.13	0.19	-0.06
116.9	100	0.267	0.10	0.12	0.16	-0.08
89.4	60	0.315	0.04	0.16	0.27	-0.13
89.4	80	0.339	0.06	0.14	0.23	-0.08
89.4	120	0.316	0.08	0.13	0.16	-0.05

mobile phases containing supercritical fluid heptane [70] and 1,1,1,2-tetrafluoroethane [20,70].

For supercritical fluid carbon dioxide at gas-like densities on an open tubular column coated with a low polarity stationary phase, changes in selectivity with variation in temperature and pressure were small, Table 7.6 [125,130]. Increasing pressure (density) at a constant temperature reduces the *l* and *s* system constants, representing cavity formation and dispersion interactions and dipole-type interactions, respectively, and increases the *r* system constant, representing electron lone pair interactions, in an approximately linear manner. Increasing temperature at a constant pressure (resulting in a decrease in density) reduces the *a* system constant and increases the *b* system constant, representing hydrogen-bond basicity and acidity, respectively, in an approximately linear manner. It can be concluded that carbon dioxide is clearly a weak solvent with a limited capacity for polar interactions. A significant contribution to the *s* and *a* system constants can be accounted for by stationary phase properties, analogous to the observed system constants for similar poly(dimethylsiloxane) stationary phases in gas chromatography. The most likely contribution that can be attributed to carbon dioxide at the prevailing mobile phase densities is changes in dispersion interactions in the mobile phase. There is some difficulty over the sign of the *b* system constant, which although small suggests that carbon dioxide is a stronger hydrogen-bond acid than the stationary phase. This is illogical of course, and was explained by attributing the negative value to weak hard Lewis acid interactions for carbon dioxide. Adding methanol to carbon dioxide at low densities resulted initially in a large decrease in the *s* system constant and a continuous reduction in the *l* and *b* system constants [126]. These results indicate that for open tubular columns, simultaneous modification of the solvation properties of the stationary and mobile phases by the modifier, play a larger role in changing selectivity than masking active silanol groups by the modifier, which is more important for packed columns containing silica-based sorbents [126-130].

The changes in system constants with composition of a binary mixture of carbon dioxide and 1,1,1,2-tetrafluoroethane on a column containing a porous polymer sorbent are illustrated by the results in Figure 7.7 [20]. Adding 1,1,1,2-tetrafluoroethane to

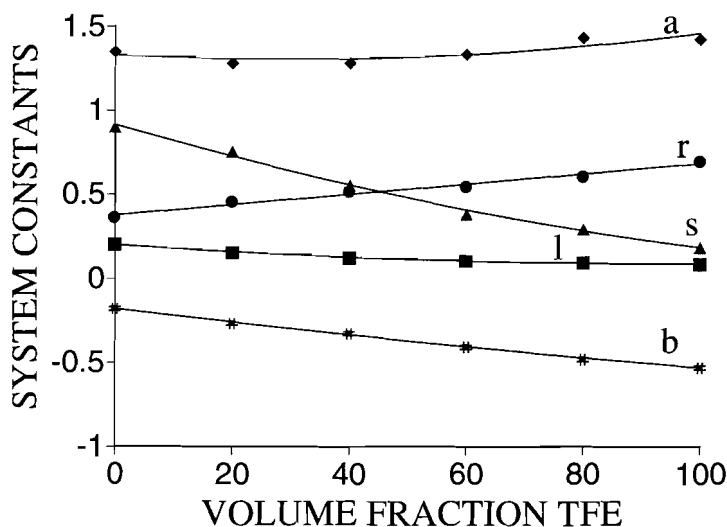


Figure 7.7. System constants of the solvation parameter model for retention on a porous polymer stationary phase with a binary mixture of carbon dioxide and 1,1,1,2-tetrafluoroethane as the mobile phase. Column 25 cm x 4.6 mm I.D. Jordi-Gel RP-C18 with a 5 μ m average particle diameter. The total fluid flow rate was 1.0 ml/min, backpressure 200 bar and temperature 125°C.

carbon dioxide influences selectivity by a near linear change in the l , s and b system constants and a modest increases in the r system constant. A significant contribution to the system constants is certainly due to the sorption properties of the stationary phase. Also, it is likely that the variable uptake of mobile phase components by the stationary phase at different mobile phase compositions is a significant contributor to the change in system constants with mobile phase composition. Consequently, the mobile phase contribution to the change in system constants in Figure 7.7 is difficult to isolate. It seems likely that 1,1,1,2-tetrafluoroethane is more cohesive, dipolar and hydrogen-bond acidic than carbon dioxide and a very weak hydrogen-bond base. The solvation parameter models show a good statistical fit to the experimental data and should be useful for estimating the retention of further compounds.

7.5.1 Programmed Techniques

The most common separation techniques in supercritical fluid chromatography employ different programmed techniques, emulating general trends in gas and liquid chromatography where temperature and composition gradients, respectively, are used routinely. The compressibility of supercritical fluids allows pressure (density) and velocity gradients to be used in addition to temperature and composition gradients. These gradients can be employed individually or in combination to reduce separation times, improve resolution, enhance sample detectability, and to extend the molecular weight

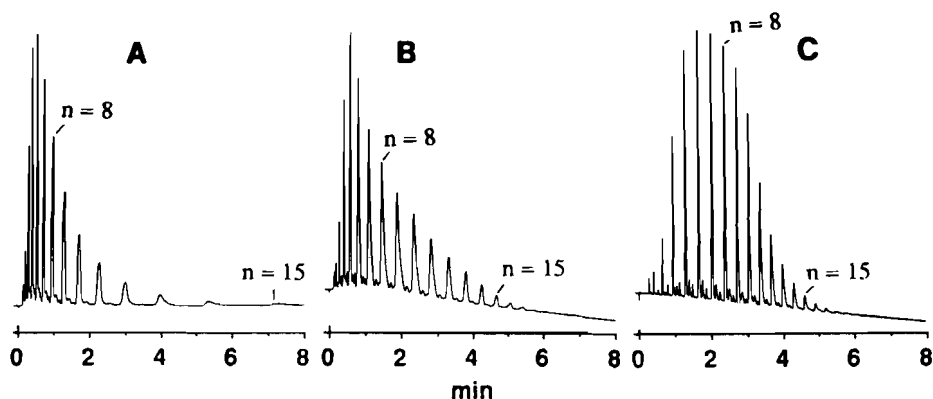


Figure 7.8. Separation of Triton X-114 by SFC using programmed elution on a 10 cm x 2 mm I.D. Nucleosil C_{18} column, 3 μ m packing, at 170°C with UV detection at 278 nm. Separation on the left: isobaric conditions at 210 bar with a mobile phase of carbon dioxide and methanol (2 + 0.125) ml/min. Center separation: composition gradient from 0.025 to 0.4 ml/min methanol over 8 min with other conditions as above. Separation on the right: pressure program from 130 to 375 bar over 8 min with the same mobile phase used for the isobaric separation. (From ref. [137]; ©Copyright Preston Publications, Inc.)

or polarity range of eluted compounds [4,120,131-138]. A typical example is shown in Figure 7.8 for the separation of a mixture of poly(ethylene glycol ether) oligomers [137]. At constant density the first few members of the oligomeric series are sharp and separated to baseline, while later members are retained by ever-increasing time increments, broadened, and increasingly difficult to detect. Programming an increase in the modifier concentration reduces the separation time and sharpens later eluting peaks. At a constant composition, programming the pressure achieves similar goals, and in this case leads to improved resolution of minor components. Pressure programming causes an increase in mobile phase density, which increases sample solubility. In this mode the type of intermolecular interactions remain largely the same and the principal observed change is due to an increase in the elution strength of the mobile phase. Composition gradients generally involve an increase in elution strength accompanied by a change in selectivity. Density and composition gradients, therefore, are complementary separation approaches.

Pressure or density programming is the most popular programmed technique for carbon dioxide and other neat supercritical fluid mobile phases. Density is the important parameter with respect to retention but pressure is the physical property directly monitored by supercritical fluid chromatographic instruments. If enough experimental density-volume-temperature data are available for the mobile phase then a computer-based algorithm can be used to generate specific density programs. Changes in linear velocity during pressure programming result from both the pressure-dependent mobile phase flow through a restrictor at the column exit (section 7.6.1.1), if installed, and the velocity gradient along the column resulting from fluid compressibility. Consequently,

pressure (density) programming is performed with a simultaneous mobile phase velocity gradient that can have several effects on the quality of the separation [139]. One difficulty is that the pressure gradient and velocity gradient are not simple functions of each other, adding to the complexity of theoretical models attempting to describe retention under different experimental conditions [135]. Although linear density programs are most commonly used, in certain circumstances nonlinear programs can provide better resolution and shorter separation times [132,134]. For example, the retention of members of a homologous series is a logarithmic function of density. Therefore, asymptotic density programming leads to a more even spacing of components for samples of this type.

One of the primary benefits of pressure (density) programming is peak compression. This results in later eluting peaks having about the same peak width as early eluting peaks. Qualitatively, this can be ascribed to either positional variation in mobile phase density or velocity along the column. Under programmed conditions the mass flow of mobile phase at the column inlet exceeds that at the outlet resulting in a velocity gradient with the velocity of the mobile phase being higher at the column inlet. Consequently, those parts of the sample zone nearest the column inlet are moving faster than the parts further down the column resulting in a compression of the sample zone. For a column with a significant density drop peak compression can occur by an increase in the retention factor ratio within the sample zone. The density gradient across the zone results in the front of the zone moving at a slower velocity than the rear leading to zone compression in the direction of migration. The peak compression effect is more pronounced the greater the density and velocity gradients along the column. At some point other competing processes that result in decreased resolution outweigh the benefits of the peak compression mechanism. At higher densities diffusion coefficients are decreased and at higher velocities the contribution of the mass transfer term increases, both leading to a loss in column efficiency. It has also been shown that large density and velocity gradients lead to a loss in column selectivity [138]. In general, therefore, to achieve optimal resolution during density programming, both the relative column pressure drop and the relative decrease in the mobile phase linear velocity should be small. In addition, a relatively slow increase in density should lead to improved resolution for difficult separations.

Positive, negative, and synchronized temperature (density) programs have been used in supercritical fluid chromatography, but less frequently than pressure (density) programs [85,132,140,141]. In pressure (density) programs high temperatures are preferred for the separation of thermally stable compounds that are soluble in low-density mobile phases. Negative temperature programs at a constant pressure lead to higher densities and has a similar effect to density programs. But since higher densities are reached at lower temperatures, column efficiency declines more noticeably. Since pressure and temperature control the maximum possible density for a particular mobile phase, and the available pressure is fixed by the design of the instrument, temperature is the only variable that can be changed to maximize the terminal mobile phase density. Synchronized density and temperature programs should provide the optimal

conditions for separating samples of a wide molecular mass range requiring a high final density to elute the last few mixture components [142]. The slow heat transfer properties of some packed columns precludes their use for temperature programmed separations.

Composition gradients are commonly used in packed column supercritical fluid chromatography [6,8,133,143-145]. Density programming is an effective tool for changing solvent strength but it has little influence on selectivity. Selectivity is easier to change using composition gradients as in liquid chromatography. Significant changes in the critical parameters of the mobile phase occur during composition programs. It is less important that the mobile phase remains in the supercritical fluid region than phase separation is avoided. Retention time stability in packed column supercritical fluid chromatography is generally better than for normal-phase liquid chromatography and column equilibration is faster. Since the viscosity of supercritical fluids is significantly less than that for liquids longer columns can be accommodated to increase the plate number for a separation or faster separations can be obtained using standard length columns. There is no comprehensive model for the prediction of retention in composition gradients for supercritical fluid chromatography, and results are less predictable than for liquid chromatography.

7.5.2 Method Development

Method development in supercritical fluid chromatography is an empirical process due to the more complex relationships between experimental variables and the greater number of variables encountered in supercritical fluid chromatography compared with gas and liquid chromatography [3,6,146]. There is no simple equation for resolution because selectivity, retention factors and column efficiency vary along the column in response to the compressibility of the mobile phase [147]. Supercritical fluid chromatography is considered a useful technique for specific problems and is unlikely to gain favor for the separation of samples that are easily obtained by gas or liquid chromatography. Compared with gas chromatography it offers lower operating temperatures and a small extension of the molecular mass separation range. Compared with liquid chromatography it can provide faster separations and a wider selection of detectors. It competes most effectively with liquid-solid chromatography but lacks access to the full range of separation mechanisms available for liquid chromatography.

Traditionally there are two complementary approaches to supercritical fluid chromatography. Separations using open tubular columns resemble an extension of gas chromatography with usually, but not exclusively, pure carbon dioxide as mobile phase, variation in mobile phase density as the primary method of increasing elution strength, and flame ionization detection for recording the chromatogram. Packed column supercritical fluid chromatography is closer to liquid chromatography with usually, but not exclusively, mixed mobile phases, composition variation as the primary variable to control elution strength, and UV detection to record the chromatogram. The instrument requirements for the two approaches are different and systems optimized for one column type

are generally unsuitable or non-optimum for the other. Open tubular columns with near optimal diameters are comparatively difficult to use and packed column supercritical fluid chromatography is considered the more robust and practical technique for routine applications. The phase ratio of typical packed columns is 10-100 times higher than for open tubular columns. This is reflected in significantly greater retention in general for packed columns, and in part explains the common use of mixed mobile phases for separations using packed columns.

Method development begins with the selection of a mobile phase, column and instrument configuration. A small number of screening experiments are then performed to assess the problem followed by trial and error optimization of the conditions identified as most favorable for the separation. The solubility of the sample in common solvents can provide some insight into possible separation conditions. Samples that can be dissolved in water or a buffer only, and are otherwise insoluble in organic solvents, are poor choices for supercritical fluid chromatography. These samples are usually better handled by other techniques, or will probably require derivatization prior to separation. If a sample is soluble in low polarity organic solvents and has limited solubility in polar organic solvents, then separation with neat carbon dioxide may be possible. If the sample is readily soluble in a wide range of solvents, including polar organic solvents, it will likely require a mixed mobile phase for separation. For samples suitable for separation using carbon dioxide either open tubular columns or packed columns with a non-polar chemically bonded phase should provide a reasonable starting point. For samples suitable for separation with mixed mobile phases carbon dioxide-methanol mixtures and a polar chemically bonded phase (e.g. 3-cyanopropylsiloxane-bonded phase) is a reasonable starting point. Other conditions for screening experiments are summarized in Table 7.7 and Table 7.8 for packed and open tubular columns, respectively.

The first experiments should be done with conditions likely to provide elution and in subsequent experiments the elution strength of the mobile phase reduced to provide a reasonable retention factor range. For neat carbon dioxide pressure programming to a high final density is a reasonable approach. For mixed mobile phases a high density, low temperature, and a high volume fraction of methanol (20-30 %) is used. Selection of the experimental variables of pressure, temperature and composition with constant elution or gradient conditions are identified from the chromatograms according to general guidelines in Table 7.7. Optimization of selectivity then proceeds by incremental changes to the experimental variables in order of their influence on selectivity. Column length can also be optimized if a small improvement in a reasonable separation is required. Failure to obtain an acceptable separation at this stage may require evaluation of a different stationary phase or mobile phase composition for packed columns or a different stationary phase for open tubular columns. Problems with poor peak shape may require the use of additives (for packed columns). Selection of a new stationary phase or mobile phase composition will often result in large changes in system properties requiring the optimization strategy to be started over again and repeated in a cyclic fashion until an accept separation is obtained.

Table 7.7

Guide to the practice of packed column supercritical fluid chromatography

Resembles liquid chromatography. Mixed mobile phases with carbon dioxide and methanol or acetonitrile are commonly used. Most useful modifier concentration range is 1-30 % with temperatures between 20-100°C. With carbon dioxide-methanol mixtures most compositions with a density < 0.5 g/ml are unstable. Additives are required to improve peak shapes and control retention for acids, bases and some polar compounds.

Initial conditions

Column	Silica gel or silica-based chemically bonded or encapsulated phases with octadecyl, octyl, phenyl, cyanopropyl, aminopropyl or spacer bonded propanediol substituents. Column length 15-25 cm with an internal diameter of 1-6 mm (4-5 mm most common) and a particle size of 5 or 10 μm . Packed capillaries: 15 cm – 2 m, internal diameter 250 μm , particle size 3, 5, 10 or 15 μm .
Non-polar solutes	Carbon dioxide, P = 80 bar, program to 350 bar at 10 bar / min, T = 80°C
Polar solutes	Carbon dioxide containing 20-30% methanol, P = 200 bar, T = 40°C
Flow rate	2.5 ml/min for a 4.6 mm I.D. column (0.5 ml/min for a 2 mm I.D. column and 0.125 ml/min for 1 mm I.D. column)
Instrumentation	Pumps operated in a flow control mode with pressure controlled by a backpressure regulator mounted downstream from the column (except for packed capillary columns). UV detector with a high-pressure flow cell or evaporative light-scattering detector for compounds lacking a chromophore

Optimization

Retention	Modifier concentration most important for controlling elution strength Pressure (density) next most important parameter Temperature least effective for adjusting retention
Selectivity	Temperature is the most useful parameter for adjusting selectivity at the optimum eluent strength Pressure (density) is the next most important parameter Modifier type has a strong influence on selectivity but offers limited flexibility Modifier concentration is the least effective variable when the retention range is optimal.
Additives	Used to minimize tailing of some polar compounds. Has a significant effect on retention and selectivity of compounds affected by the additive. Additives (e.g. trifluoroacetic acid or isopropylamine) are used at a concentration of 0.1-1% in methanol. Strong acids (bases) are used to improve the peak shape of weak acids (bases). Steric hindrance minimizes the effectiveness of tertiary and quaternary amines as additives.

Table 7.8

Guide to the practice of open tubular column supercritical fluid chromatography

Resembles high-pressure gas chromatography. Pressure (density) programming generally used to change elution strength (temperature programming less common). Typical temperatures > 80°C. Carbon dioxide alone or possibly saturated with water or formic acid as a mobile phase. Mixed mobile phases rarely used.

Initial conditions

Column	Crosslinked poly(siloxane) with methyl, octyl, phenyl, biphenyl, or cyanopropyl substituents. Column length 2-10 m with an internal diameter of 25-100 μm (50 μm most common) and film thickness < 1.0 μm (0.25 μm most common). Mobile phase velocity 10 u_{opt} or about 1-4 cm/s.
Mobile phase	Carbon dioxide, P = 80 bar, program to 350 bar at 10 bar / min, T = 80°C
Instrumentation	Flow control by a fixed restrictor between the column and detector. Flame ionization detector is generally used (UV detector when organic modifiers are used). Split injection usually required. Syringe pumps commonly used (eluent flow rate 10s $\mu\text{l/min}$).

7.6 INSTRUMENTAL ASPECTS

Most contemporary instruments for supercritical fluid chromatography are optimized for either open tubular or packed column separations and may provide a poor compromise for column types other than those they were designed for [146]. The small volumes and low flow rates associated with open tubular columns make greater demands on instrument design than typical packed columns, which have dimensions similar to those commonly used in liquid chromatography.

The fluid delivery system consists of one or more pumps and associated components to deliver single or mixed mobile phases at a controlled pressure and (preferably) constant flow. Pressure (density) and/or composition programming is usually provided for by most systems. Rotary injection valves, similar to those used in liquid chromatography, are the basis of most sample introduction system. For open tubular columns, the column oven and flame-based detectors are similar to those used in gas chromatography. For packed columns, a wider range of gas chromatography-like, Peltier, and recirculating baths are used as column ovens. For open tubular columns a temperature operating range of 50 -250°C is reasonable, while packed columns are rarely operated at temperatures above 150°C, and sub-ambient temperature capability may be useful for some separations, such as enantiomers. Temperature programming can be used with open tubular, packed capillary and small bore packed columns but is not normally used with conventional diameter columns. With pressure control pumps a restrictor is usually placed between the column and detector for flame-based detectors and after the flow cell for optical detectors. Only a small number of companies manufacture supercritical fluid chromatographic instruments for analytical separations. In spite of this, general instrument design has reached a reasonable level of maturity, and contemporary instruments offer improved performance compared to instruments available only a decade ago.

7.6.1 Fluid Delivery Systems

Modern instruments for packed column supercritical fluid chromatography use reciprocating-piston pumps (section 5.2.2) operated as flow sources in front of the column and an electromechanical backpressure regulator downstream of the column for independent pressure control, Figure 7.9 [6,7,146,148,149]. Compared with reciprocating-piston pumps used for liquid chromatography, modification is required to avoid cavitation and flow-rate variability with highly compressible fluids, such as carbon dioxide. Carbon dioxide is pumped as a liquid by using pump head chillers to maintain the carbon dioxide well below its critical point (e.g. -10 to -20°C). Even as a liquid, carbon dioxide requires pumps with an extended compressibility range, and the ability to dynamically change compressibility compensation as the pressure, temperature and volume of fluid in the pump head changes. Compensation for small leaks and adiabatic heating of the fluid as a result of piston motion is also required if an accurate and stable volumetric flow is to be achieved. Compensation for compressibility is achieved by a network of sensors interacting with appropriate pump algorithms to bring about

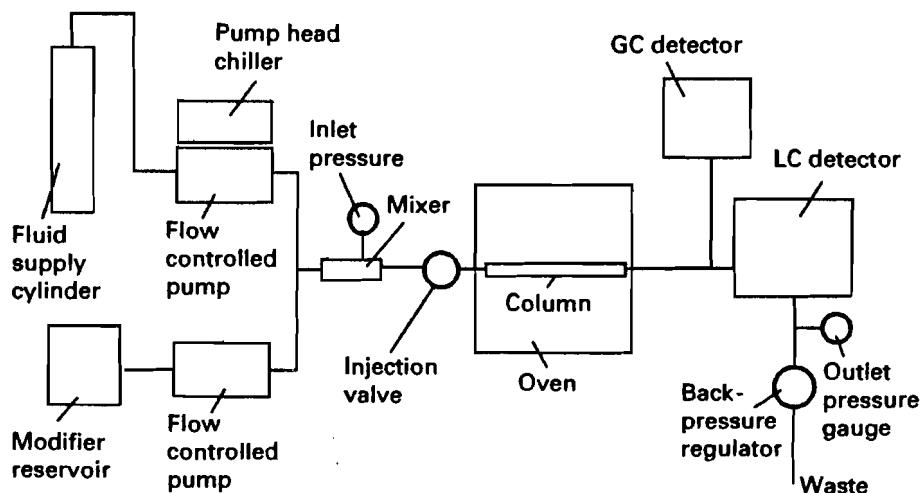


Figure 7.9. Schematic diagram of a typical packed column SFC instrument capable of pressure (density) and composition mobile phase programming. (From ref. [146].©Academic Press).

rapid changes in the pump motor speed. This feature was missing from pumps commonly used in supercritical fluid chromatography until recently. It was one of the main reasons for poor retention reproducibility of early supercritical fluid instruments. An important second component is the electromechanical backpressure regulator, which allows pressure to be set independent of flow rate [43]. In this division of duties the pump is responsible for flow control alone and the regulator for establishing the pressure at the column outlet. Earlier instruments used mechanical devices that provided a fixed pressure only but modern instruments use small volume, heated and electronically variable regulators (or nozzles, valves, etc.) in combination with a pressure transducer that provide more accurate pressure control and allow pressure to be programmed independent of the mobile phase composition. To generate typical capillary column flow rates reciprocating-piston pumps are used with a buffer volume and flow splitter [146]. A prototype reciprocating-piston pump with gradient capability for use with packed capillary columns has been described [150].

Because of the difference in compressibility between carbon dioxide and organic solvents low-pressure mixing devices are unsuitable for programming composition gradients. High pressure mixing is used with one pump suitable for pumping compressible fluids and a second regular pump for the modifier. A mixing chamber in front of the sample inlet is required for homogenizing the mobile phase. In combination with a backpressure regulator, properly designed systems allow independent control of flow, composition and pressure in packed column operation.

Syringe pumps (section 5.2.2) are widely used with open tubular and packed capillary columns and occasionally with small bore columns having internal diameters less than 2 mm [3]. Typical syringe pumps have cylinder volumes of 100 or 250 ml and lack the

reservoir capacity for convenient use with packed columns having an internal diameter greater than 2 mm. Syringe pumps can be difficult to fill with liquid carbon dioxide and chilling the pump cylinder by wrapping with cooling coils connected to a recirculating liquid bath can be helpful. Filling is improved using cylinders with a helium overpressure but this approach is not recommended due to the influence of dissolved helium on retention reproducibility (section 7.2). Syringe pumps can be operated in a constant pressure or constant volume mode but are typically used as a constant pressure source with flow controlled by a restrictor (section 7.6.1.1) placed downstream of the column. Simultaneous measurement of the column temperature and inlet pressure allows constant density or density program operation under computer control if the appropriate fluid isotherms are known or can be approximated. For carbon dioxide this is not a problem, but the relevant data and/or programming may not be available for other mobile phases. When using syringe pumps the algorithm controlling pump operation must also accommodate temperature changes resulting from expansion or contraction of the fluid volume in the syringe pump during use. This is relatively simple to allow for with well-characterized mobile phases, such as carbon dioxide, but may not be accommodated by the pump controller for other single or mixed mobile phases.

Mixed mobile phases and composition gradients are less commonly used in open tubular column supercritical fluid chromatography. Mixed mobile phases of a fixed composition are commercially available or can be prepared by adding the modifier to the syringe cylinder and filling the remainder of the cylinder with liquid carbon dioxide. The composition of premixed cylinders varies with the volume of liquid remaining in the cylinder and is unsuitable for most applications that require reproducible retention [151]. Composition gradients can be generated using two syringe pumps and a mixing chamber. Poor gradient composition accuracy and flow stability due to the effect of fluid compressibility limit this approach [3]. For small bore columns reasonable gradient reproducibility was obtained using a syringe pump to deliver liquid carbon dioxide and a reciprocating-piston pump to add the modifier [152]. For the low mixing ratios typical of capillary column flow rates the modifier pump is required to operate at flow rates of 0.1 to 1.0 $\mu\text{l}/\text{min}$. A better approach uses a pressurized modifier reservoir and an air actuated high-pressure valve for the controlled addition of variable amounts of modifier to liquid carbon dioxide [153]. A number of approaches using a single pump and on-line parallel addition of modifier using capillary tubes of different size as modifier reservoirs have been used to create step or continuous gradients [154,155]. The modifier is added to an empty capillary tube. A portion of the liquid carbon dioxide delivered by the pump passes through the reservoir displacing the modifier while the other part bypasses the reservoir. The two streams are recombined at a mixing tee. In principal continuous gradients can be obtained by varying the relative flow rates of the two streams.

7.6.1.1 Restrictors

A restrictor is required to maintain supercritical fluid conditions along the column and to control the linear velocity of the mobile phase through the column when the column

inlet is pressure controlled. A restrictor is commonly used with syringe pumps and usually when open tubular and packed capillary columns are used. Restrictors also provide an interface for gas phase detectors. The restrictor is positioned between the column and the detector and is responsible for the effective transfer of the column eluent from the supercritical fluid phase to the gas phase. The restrictor should have a low dead volume and minimize particle formation, which leads to spiking in the detector signal.

The ideal restrictor should be inert, immune from plugging, adjustable, easily replaceable, and effective for all sample types [135,156]. There is no ideal restrictor. Commonly used devices include crimped tubing [54,144,157-159], linear [156,160,161], robot-pulled tapered [161-163], integral [160,164,165], frit [156,166] and multichannel [167,168] restrictors. For spectroscopic detectors with high-pressure cells the restrictor is placed after the detector and relatively simple crimped tubing, mechanically adjustable valves, or pneumatic systems can be used at packed column flow rates with few problems. Density programming with a constant linear velocity is possible by controlling the outlet pressure with a programmable electromechanical regulator. A programmable backpressure regulator based on a sheath-flow nozzle was described for open tubular columns [159]. However, fixed restrictors are more commonly used with open tubular and packed capillary columns, particularly when flame-based detectors are used.

Linear restrictors consist of a short length of fused-silica capillary tubing (10-15 cm long and 5-25 μm internal diameter). They perform adequately with low molecular mass compounds, but produce detector spikes with polar and high molecular mass compounds resulting from formation of fog particles during decompression. In flame-based detectors these particles create short bursts of ions registered as spikes in the detector signal. A solution to this problem is to carry out the decompression over a short distance and to ensure that the restrictor tip is supplied with sufficient heat to avoid condensation of the sample. Tapered and integral restrictors fit into this category. The internal diameter of a tapered restrictor is reduced from 25-50 μm to 2-5 μm over the final 1-3 cm of the capillary tube. The taper allows decompression over a short distance, while the thin wall at the tip allows greater heat transfer to the expanding effluent jet, both of which contribute to the elimination of spiking. Tapered restrictors are rather fragile and prone to break unless fitted with a protective fused-silica sleeve [163]. Integral restrictors are less fragile than tapered restrictors. They can be simply prepared (after some practice) by first closing the end of a suitable fused-silica capillary in a flame and then sand papering back the tip to produce a pin hole of 1-2 μm . The integral restrictor provides virtually point restriction but flow rate adjustment is often difficult and the orifice is prone to plugging. The multipath frit restrictor is prepared by bonding a 1-3 cm inorganic porous frit inside a short length of fused-silica capillary tubing and is more durable than the tapered restrictors. The multiple flow paths and longer residence time of the mobile phase within the frit region allows more efficient heat transfer minimizing spiking. Frit restrictors exhibit unacceptable performance for the separation of high molecular mass samples at pressures exceeding 400 atmospheres. The integral restrictor is a better choice for these samples and extreme pressures, Figure 7.10 [156].

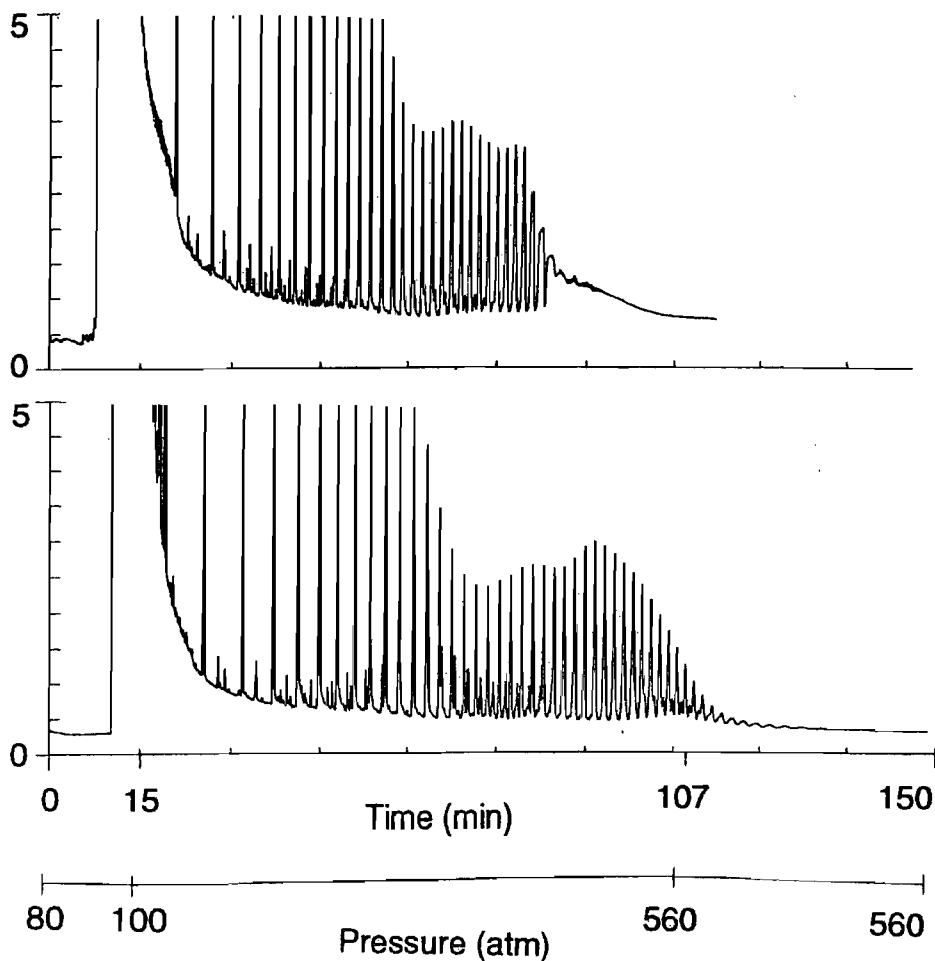


Figure 7.10. Separation of a broad molecular mass poly(ethylene glycol) trimethylsilyl ether sample using a frit restrictor (top) and integral restrictor (bottom). Column: 10 m x 50 μm I.D. SB-biphenyl-30, film thickness 0.25 μm connected to the injector by a 1 m x 0.50 μm uncoated retention gap. Conditions: Temperature 120°C; pressure program 80 atm to 100 atm at 1.3 atm/min then 100 atm to 560 atm at 5 atm/min and held at 560 atm for 80 min; mobile phase carbon dioxide. (From ref. [156]. ©Wiley-VCH).

The multichannel restrictor is fabricated from a short glass plug sealed into a fused-silica capillary tube. The glass plug is permeated by parallel channels 1–2 μm in diameter. The number, length and diameter of the channels can be varied to produce restrictors with different flow characteristics.

Sometimes it is desirable to operate a supercritical fluid chromatograph with two detectors. If one of these detectors is a low or atmospheric pressure detector, such

as a mass spectrometer, evaporative light-scattering detector or any of the flame-based gas chromatographic detectors, some modification to the general arrangement for pressure and flow control of the column is required. For a typical packed column with pressure controlled by a backpressure regulator downstream of the column three options are possible [148,169]. The detector can be connected directly to the outlet of the backpressure regulator through a capillary tube. This solution is the simplest but generally the least satisfactory. The transfer tube is a source of dead volume and poor control of the pressure and temperature of the decompressed mobile phase in the transfer tube may result in precipitation of sample components. A more favorable solution is to insert a splitter tee between the column and the regulator. A portion of the column eluent is split to the low-pressure detector through a restrictor. The only disadvantage of this approach is that the response of the low-pressure detector depends on the split ratio, which may not be constant during programmed separations. An alternative approach is to replace the pressure regulator with a pressure-controlled pump. The pump is used to introduce a pressure-regulating solvent into the tee between the column and the detector with a flow restrictor at the detector inlet. The advantage of this approach is that the restrictor affects only the flow rate of the pressure-regulating fluid and the response of mass-sensitive detectors is not compromised. For open tubular columns, a splitter tee with parallel flow linear restrictors (10 cm x 14 μ m I.D.) was used for simultaneous operation of low-pressure and high-pressure detectors [170]. The flow rate through each restrictor was controlled by independently changing its temperature.

7.6.2 Sample Inlets

Nearly all sample introduction techniques in supercritical fluid chromatography use high-pressure rotary valves identical to those used in liquid chromatography (section 5.3.1) [3,4,146,149,171-173]. These valves are used with modifications to the valve-column interface to accommodate the wide range of column diameters, flow rates, sample capacity and the phase behavior of supercritical fluid-liquid mixtures. Valve injection for conventional packed columns (10-20 μ l) and small bore columns (0.2-1.0 μ l) is straightforward with few problems except, perhaps, for incomplete mixing of the sample solvent with the mobile phase prior to the sample plug entering the column. This can result in excessive band broadening and even peak splitting. The total column volume for typical open tubular columns used in supercritical fluid chromatography is no more than a few microliters. Acceptable sample volumes are in the nanoliter range, placing severe demands on the performance of the injection device. To overcome these problems, a wider range of injection techniques is used in open tubular column supercritical fluid chromatography [171-173]. Each represents a different compromise more suitable for some sample types than others, and no universal injection technique exists.

7.6.2.1 Packed Column

Direct injection using a rotary injection valve is the method of choice for packed columns [3,149,172]. These valves may be operated manually, but more commonly

electronic or pneumatic actuators are used. The sample loop is loaded with a solution of the sample in an organic solvent, the valve switched to the inject position, and the sample displaced into the column by a high-pressure liquid that subsequently becomes a supercritical fluid upon entering the column oven. The injection valve is normally mounted on top of the oven at a temperature close to room temperature while the column may be at a much higher temperature. Secondary cooling may be used to maintain the valve temperature close to room temperature. The sample solvent must be able to dissolve the sample at a relatively low temperature. The sample must remain soluble in the mixture of mobile phase and sample solvent as it mixes before becoming supercritical. Failure to maintain the sample in solution can result in memory effects and inaccurate quantification.

Normal injection volumes provide adequate sample amounts for most packed column applications but solvent effects sometimes result in band broadening and peak splitting [149,173-177]. The sample solvent is gradually diluted with mobile phase as it migrates through the connecting tube between the injector and column. In most cases it will be subjected to a temperature gradient since the column oven temperature generally exceeds the injection valve temperature. The critical constants and elution strength of organic solvents are greater than for carbon dioxide, and even if the sample solvent and mobile phase are well mixed, the sample may still be delivered to the column in a mixture of liquid and fluid phases. This has the effect of delivering at least part of the sample to the column in a strong solvent that is not adequately focused by the column. These adverse sample introduction effects can be quite conspicuous with small bore columns and supercritical fluid carbon dioxide as the mobile phase. The higher sample capacity of conventional diameter columns, their higher flow rates, and general use of mixed mobile phases combine to overcome these injection problems in most cases. With carbon dioxide as the mobile phase an increase in the volume of the connecting tube between the injector and column to provide effective dilution of the sample solvent in the mobile phase is a possible remedy for solvent effects. Even under these conditions the sample may still be delivered to the column in a mixture of solvent and fluid that is too strong to avoid band broadening entirely. If the type of sample solvent can not be optimized to minimize the problem then solventless injection may be a better option.

Compared with the samples typically separated by supercritical fluid chromatography most solvents are significantly more volatile and can be removed from the sample by evaporation in a short open tube or packed precolumn [174-178]. Solventless injection requires only minor modification to a standard rotary injector, either addition of a second valve or tee piece and a precolumn. This allows sequential programming of the independent processes of solvent removal and sample deposition in the precolumn followed by dissolution of the sample in the mobile phase and its transport to the column for refocusing. Quite large solvent volumes (hundreds of μl) can be handled in this way, but typically smaller volumes are used. The only real limitation to solventless injection is that the volatility difference between the sample and solvent must be sufficient for effective removal of the solvent without loss of sample. In addition, effective sample focusing at the head of the column is required to maintain an acceptable separation

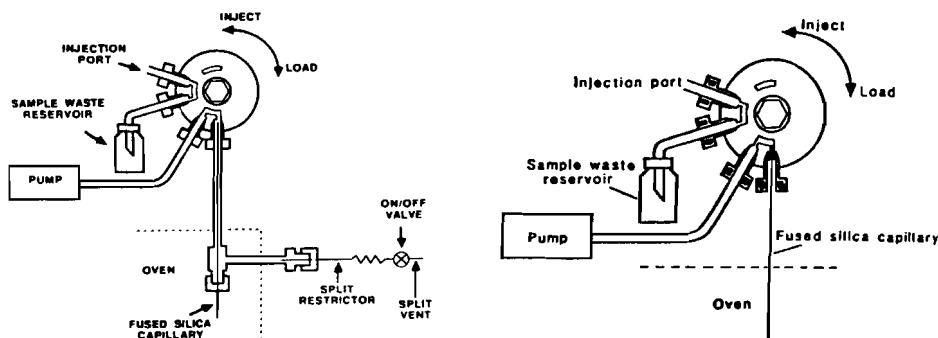


Figure 7.11. Schematic diagram of a split valve injector (A) and a timed-split injector (B) for open tubular column supercritical fluid chromatography.

quality. The retention power of the stationary phase at the mobile phase density used for sample transfer causes focusing of the sample as a result of the reduction in migration velocity which occurs as the sample enters the separation column. Pressure trapping requires a sudden decrease in density of the transfer solvent resulting in precipitation of the sample at the head of the column or an intensification of stationary phase interactions. This is easily achieved if the system pressure during the transfer process is close to the critical point of the mobile phase and an abrupt change in temperature exists between the precolumn and the column oven. After sample focusing the system parameters are abruptly changed to those required for the separation.

7.6.2.2 Open Tubular Column

Since the volumes delivered by valve injectors with the smallest available loops (60 or 200 nl) are comparatively large compared to the capacity of typical open tubular columns, split injection techniques are commonly used [171-173,179]. A typical arrangement for split injection using a valve is shown in Figure 7.11. In the dynamic split mode the valve is turned to the inject position and part of the sample enters the column with the remainder exiting via the vent line. For this type of injection the relationship between peak areas and the split ratio is nonlinear. Also, the split ratio is observed to change with sample viscosity, mobile phase density, and any time the vent flow resistance is disturbed. Dynamic split requires rather concentrated samples for a reasonable detector response and is unsuitable for trace analysis. Optimum conditions include a high split line flow (split ratio 1:100 and split line velocity 0.5-2 cm / s), short injection time (about 10 s to avoid the solvent front trailing into the separation) and concentrated samples (0.5-1.0 mg / ml to overcome the low sample utilization). Results for injection reproducibility vary widely but are rarely better than 2-18 % RSD. An internal standard is required for quantification.

An alternative form of split injection is provided by the timed split technique, Figure 7.11 [171-133,179]. In this case the column is connected directly to the valve

body and the valve electronically controlled to position the sample loop in the mobile phase flow path for a short and accurately defined time. Timed split allows variable volumes to be injected by changing the time the sample loop is connected to the column flow path but its most important characteristic is the (virtual) elimination of the solvent tail from the chromatogram. It offers more reproducible injections than the dynamic split technique but suffers from many of the same problems, namely, poor accuracy, split ratios that depend on system and sample solvent properties, and poor detection limits. Injection repeatability may be better than for dynamic split but reported results vary widely.

The delayed split method uses the same configuration as the dynamic split except for the addition of an on/off valve in the vent line [171-173]. With the split vent valve in the off position, the sample is injected in the normal way at a selected pressure. After a prescribed delay time the injection valve is returned to the load position and the vent valve opened, which affects an immediate purge of the injection area. The amount of sample transferred into the column depends on the valve actuation time and the delay time. The delayed split injection techniques can be modified for solvent backflushing [171]. Immediately after the vent valve is opened a rapid negative pressure ramp is initiated. This causes a reversal of flow in the first part of the column and precipitation of the sample on the column wall. The solvent, and probably some of the sample, is backflushed out of the column and through the open vent line. The injection repeatability is similar to timed split injection.

To allow larger sample volumes to be injected onto open tubular columns without degrading column performance various direct injection techniques employing a retention gap [171-173,180-183] or gas phase solvent venting [171-173,184-186] have been developed. Direct injection of larger volumes (0.1 to 1.0 μl) is usually accomplished by valve injection into a retention gap of sufficient length to accommodate the sample volume. A short length of the retention gap is left outside the oven to act as a damper for the pressure pulse caused by actuation of the injection valve. Initial chromatographic conditions are set so that the mobile phase or sample solvent remain a liquid at room temperature. The sample solvent must be miscible with the mobile phase. Phase separation is accomplished at constant pressure by the change in temperature as the sample solution enters the portion of the retention gap residing in the column oven. The continuous movement of solution into the oven results in the formation of a dynamic liquid film, coating the walls of the retention gap to a length that depends on the experimental conditions. It is important that the dynamic liquid film (flooded zone) does not extend into the separation column. The liquid film acts as a temporary stationary phase retaining the sample components until the sample solvent is fully removed by evaporation or selective dissolution in the mobile phase. The sample is then refocused at the head of the column by either phase ratio focusing or pressure trapping. Optimization of injection conditions is more fully described in [182]. Solvent venting techniques rely upon injection of the sample into a short empty or packed precolumn followed by removal of the solvent by gas-assisted evaporation or selective dissolution in low-density carbon dioxide. The solvent vapors are vented from the system by a valve positioned between the

precolumn and the separation column. This avoids the long time required to transport the solvent through the separation column. The sample components are then dissolved in the mobile phase and refocused at the head of the column by phase ratio focusing or pressure trapping. The general literature does not indicate that these large volume injection techniques are widely used at present.

7.6.2.3 SFE-SFC

Supercritical fluid extraction (SFE) can be coupled to SFC using a series of switching valves and either a loop or an accumulator trap interface [79,172,174,187-192]. The loop interface is used with a closed-loop static extractor, sometimes equipped with a recirculating pump. The fluid from the extraction cell passes continuously through the injection valve loop and back to the extraction cell. Injection of an aliquot of the extract onto a packed column is made by periodically switching the loop so that it is in-line with the flow of mobile phase to the separation column. This approach is used to determine fundamental parameters of the extraction process more so than for analysis.

Dynamic extraction usually provides faster and more complete extraction of most analytes. The accumulator interface allows the extract and fluid to be separated in a cryogenic or sorbent trap which is subsequently flushed with mobile phase or rapidly heated to transfer the extracted material to the separation column. Typical traps are short lengths of metal or fused-silica tubing, coated fused-silica tubing or sorbent-filled traps. The extractor is connected to the accumulator trap via a capillary restrictor. During extraction the depressurized gas from the restrictor passes through the trap and is vented to atmosphere. Analyte retention in the trap depends on the trap dimensions, temperature, pressure and sorbent type, if used. Traps cannot be too large because of problems this causes in focusing analytes at the column. External cooling by expanding carbon dioxide to -50 to 0°C is commonly used with open tubular traps to increase the recovery of volatile analytes. The trap temperature, however, should be no lower than the temperature required for efficient retention of the analytes. Too low a temperature may result in increased restrictor plugging or blockage of the trap by condensed matrix components, such as water. Independently heated linear restrictors are often used to minimize plugging and to provide a predictable range of fluid flow rates for extraction. Modified fluids can be used but the trapping conditions must be optimized to avoid condensation of the solvent.

Major applications of SFE-SFC are somewhat limited at the moment to the analysis of lipids and pesticides from foods and similar matrices and different types of additives used in the production of polymers [79,146,188-194]. The approaches used cover a wide range of sophistication and automation from comprehensive commercial systems to simple laboratory constructed devices based on the solventless injector [172,174,175,188]. Samples usually consist of solid matrices or liquids supported on an inert carrier matrix. Aqueous solutions are often analyzed after solid-phase extraction (SPE-SFE-SFC) to minimize problems with frozen water in the interface [178,190]. The small number of contemporary applications of SFE-SFC reflects a lack of confidence in supercritical fluid chromatography as a separation technique and competition for

supercritical fluid extraction from a number of new extraction processes, some of which provide more favorable extraction properties.

7.6.3 Coupled-Column Systems

Series coupling of columns containing the same stationary phase is used to enhance efficiency and with different stationary phases to fine tune selectivity [8]. Series coupling of packed columns became popular after it was demonstrated that the column pressure drop did not limit the total column efficiency to the extent that had been predicted (section 7.4.2). Serial coupling of 2 or 3 standard columns is practical for routine applications and provides a total plate count in excess of 50,000. There is no theory for selectivity optimization for coupled packed columns but suitable conditions often can be estimated from separations on the individual columns. Effective selectivity changes require that the coupled columns have different retention properties. A number of practical examples for the separation of polymer additives, polycyclic aromatic hydrocarbons, phytic acid impurities and enantiomers have been described [137,195,196]. Series coupling of open tubular columns with different stationary phases is less common, but changes in selectivity are predictable, at least when the pressure drop is low [197].

With series coupled columns the mobile phase is the same for all columns. Multidimensional separations allow independent optimization of the mobile and stationary phases but limit the improvement in the separation to those portions of the first column separation isolated and transferred to the second column. Approaches to packed column multidimensional supercritical fluid chromatography are similar to those used in liquid chromatography (section 5.6.1) and for open tubular columns similar to those used in gas chromatography (section 3.8.1). Conventional packed columns are coupled through a valve switching interface, in which the components of interest are diverted to the second column or isolated in a separate loop and subsequently injected into the second column [8,198,199]. The automated separation of gasoline and middle distillate fuels into saturates, olefins and aromatics using three packed columns containing different stationary phases and a valve switching interface has been reduced to routine practice in the petroleum industry [200-202]. For coupling packed capillary columns or coupling a packed capillary column to an open tubular column a flow switching interface with a cryogenic trap for focusing the fractions transferred from the first column has been described [203]. This interface is also suitable for coupling open tubular columns [204] as well as an offset-cross interface that functions similar to live switching in multidimensional gas chromatography [205]. Coupled open tubular columns provide higher resolving power than packed columns but separation times are very long. A suitable compromise between resolution and separation time is reached by coupling a packed capillary column to an open tubular column for separating difficult mixtures. Using a packed column for the first dimension also increases the sample loading capacity improving the prospects of detecting minor components in the fractions transferred to the second column. Often an acceptable

alternative to on-line multidimensional supercritical fluid chromatography is automated semipreparative supercritical fluid chromatography with fraction collection to isolate extracts for subsequent analysis [8]. This approach provides greater flexibility and allows a higher sample loading for the fractionation step.

7.6.4 Detectors

Most gas and liquid chromatography detectors can be used with little modification for both packed and open tubular column supercritical fluid chromatography. Liquid chromatography detectors usually require a high-pressure flow cell for use with packed columns and a degree of miniaturization for open tubular columns. Gas chromatography detectors can be used without modification with open tubular and packed capillary columns but some allowance for the higher flow rates of conventional packed columns (> 1000 ml / min as gas) must be made. In supercritical fluid chromatography liquid phase detectors are located before the restrictor and gas phase detectors after the restrictor. The most popular detectors are the UV detector (section 5.7.1) for packed columns and separations using mixed mobile phases, the flame ionization detector (section 3.10.1) for open tubular and packed capillary columns with carbon dioxide as a mobile phase, and the evaporative light-scattering detector (section 5.7.3) for all column types.

Carbon dioxide is transparent at all wavelengths down to < 210 nm. Interfacing a UV detector with a high-pressure flow cell to standard packed columns requires no special arrangements [3,202,206,207]. Changes in temperature within the flow cell result in noisy baselines and a degradation of detector performance as a result of refractive index effects. Heat exchangers provided with UV detectors for liquid chromatography may not be adequate for temperature control in supercritical fluid chromatography where a large temperature difference between the column and detector flow cell may exist. Open tubular and packed capillary columns place stringent limitations on the detector cell volume, which are usually met using on-column or pseudo on-column flow cells, similar to those used for packed capillary liquid chromatography (section 5.7.1). One shortcoming of these cells is baseline drift during pressure programming caused by density related retention index changes. These effects can be minimized by using detectors with forward optics that place the detector cell close to the sensor [207]. A fluorescence detector with a standard flow cell was interfaced to a packed column supercritical fluid chromatograph using a restrictor before the flow cell for decompression of the mobile phase and a backpressure regulator at 50 bar after the cell to maintain the mobile phase in the liquid state in the flow cell [208]. A scanning fluorescence detector with a fiber optic design was described for use in open tubular column supercritical fluid chromatography [209]. A radioactivity detector with a flow through cell was coupled to a packed column SFC with few problems [210].

The flame ionization detector shows virtually no response to carbon dioxide but is generally incompatible with mixed mobile phases containing organic solvents except for water and formic acid [3,166,211]. Apart from a small reduction in sensitivity and

incompatibility with the high flow rates of conventional packed columns (a postcolumn splitter is used in this case, Figure 7.9), the flame ionization detector is every bit as easy to use in supercritical fluid chromatography as it is in gas chromatography. Other gas chromatographic detectors that have been used in supercritical fluid chromatography include: the thermionic ionization detector [166,212]; flame photometric detector [3,213]; photoionization detector [3,166]; electron-capture detector [166,214,215]; chemiluminescence sulfur [216,217] and nitrogen [218] detector; atomic emission detector [219,220]; and inductively coupled plasma atomic emission (or mass spectrometry) detector [221]. Evaluation of these detectors is not extensive indicating limited use. The parameters normally selected for optimization are the ratio of the detector gas/makeup gas/decompressed mobile phase gas flow rates, temperature, position of the restrictor, and the identity and amount of any modifier. The detector base supplies heat to the restrictor tip to avoid plugging. The design of the detector base is important since uncontrolled heating of the restrictor can cause precipitation of analytes by decreasing the fluid density in the transfer line. Although detection limits are usually higher than those observed for gas chromatography and heteroatom selectivity is degraded to some extent, operation of these detectors is not particularly difficult.

The evaporative light-scattering detector for liquid chromatography is suitable for use with packed columns in supercritical fluid chromatography. Its performance can be improved, however, by minor modifications [8,50,137,222]. A miniaturized detector is required for optimal performance with open tubular and packed capillary columns [8,81,223,224]. Several different nebulizer designs have been utilized in packed column supercritical fluid chromatography. In most cases a liquid chromatographic nebulizer with a restrictor inserted into the nebulizer assembly is used. The performance of the detector is improved by heating the region close to the restrictor tip and adding makeup gas flow to provide an optimized total flow of expanded supercritical fluid and makeup gas. In this way interference in the detection step from the formation of dry ice and water ice particles are minimized. The detector can be connected to the supercritical fluid chromatograph using a splitter interface, or by connecting the restrictor to the outlet of a single or two-stage backpressure regulator. A secondary pressure-controlled solvent flow added at a tee between the column and restrictor can be used for pressure control of the column outlet, and to allow independent optimization of the operating flow rates of the column and detector, as well as minimizing ice formation during the depressurization process [148]. The detector is generally reliable in routine operation, is suitable for use with density and composition programming, and provides low nanogram detection limits for solutes of low volatility.

7.7 RELATED TECHNIQUES

7.7.1 Enhanced-Fluidity Liquid Chromatography

Enhanced-fluidity mobile phases are prepared by dissolving a large amount of a low viscosity liquefied gas, such as carbon dioxide or fluoroform, in associated liquid

solvents. Binary mixtures containing a large mole fraction of carbon dioxide can be formed with most common organic solvents. At room temperature liquid carbon dioxide is poorly soluble in water and requires a cosolvent such as methanol for the preparation of ternary mixtures. The total amount of carbon dioxide in ternary mixtures containing water is controlled by the amount of water present. For example, a 70:30 methanol-water mixture is miscible with up to about 0.5 mole fraction of carbon dioxide.

Enhanced-fluidity liquids combine the advantages of low viscosity and high sample diffusivity at room temperature with the high solvent strength of liquids. These properties are reflected in higher efficiency, a higher optimum mobile phase velocity, shorter separation times, and a lower column pressure drop (facilitating separations requiring long columns) when compared with typical mobile phases used for liquid chromatography. Applications to normal-phase [225], reversed-phase [225,227-229], size-exclusion [224,230], and liquid chromatography at the critical adsorption point [231] have been described. Addition of carbon dioxide to mobile phases containing water results in the formation of carbonic acid [228,229]. Therefore, acidic but not basic buffers can be used for reversed-phase separations of ionizable compounds when carbon dioxide and water are components of the mobile phase. The equipment used for enhanced-fluidity liquid chromatography is identical to packed column supercritical fluid chromatography. Important considerations are provision for pumping highly compressible liquids such as carbon dioxide, high pressure mixing for the preparation of binary and ternary mobile phases, and pressure control of the column outlet to maintain single-phase conditions.

7.7.2 Separations with Superheated Water

In reversed-phase chromatography water is a weak eluent at room temperature (section 4.3.1). Supercritical fluid water is too reactive to samples and system components alike to be practically useful as a mobile phase, and requires extreme temperature and pressure conditions to maintain the mobile phase in the supercritical region, Table 7.3. Under less extreme conditions superheated water (also called pressurized hot water or subcritical water) is capable of useful reversed-phase separations without the use of organic solvents [232]. When water is heated under pressure its dielectric constant, cohesive energy, viscosity and surface tension decrease. At temperatures from 100 to 250°C the elution strength of superheated water in reversed-phase chromatography is similar to conventional water-organic solvent mixtures [232,233]. The elution strength of superheated water at 180°C on a porous polymer sorbent is similar to 50-60 % (v/v) methanol or 15-25 % (v/v) acetonitrile in water at room temperature. General trends in retention are for higher temperatures to reduce retention due to a reduction in the cohesive energy of water [233]. A selective increase in retention is observed for hydrogen-bond bases arising from a decrease in the hydrogen-bond acidity of water at higher temperatures. Plots of the retention factor against temperature (section 1.4.4) are sometimes non-linear over an extended temperature range for superheated water [233,234]. Programming temperature for

superheated water systematically increases eluent strength similar to composition gradients, but the range of retention factor reduction is less than that possible for composition gradients [233,235-237]. Inorganic buffers can be used for pH control in superheated water without problems [237,238].

Instrumentation for superheated water mobile phases makes use of components from gas and liquid chromatography. Pumps and injection valves are identical to those used for liquid chromatography. Gas chromatography ovens have generally been used for temperature control because of the high temperatures commonly used (100-250°C) and the general interest in temperature programming as a method of gradient elution. A length of capillary tube heated by the column oven and positioned between the injection valve and the column is used to preheat the mobile phase entering the column to minimize axial temperature gradients, which can result in distorted peak shapes and reduced efficiency. A 1.5 m x 0.17 mm I.D. coiled capillary tube (volume = 34 μ l) is adequate for thermal equilibration of conventional diameter columns with flow rates of 0.7 to 1.5 ml / min [234]. Conventional and packed capillary columns with porous polymer, porous graphitic carbon and polymer-coated zirconia sorbents are used for separations [232-234,239,240]. Porous polymer and porous graphitic carbon sorbents are significantly more retentive than polymer-coated zirconia sorbents and generally require higher temperatures for elution of the same compounds. There are mixed reports of the suitability of silica-based octadecylsiloxane-bonded sorbents for superheated water separations [232,239]. The solubility of silica in water increases significantly at elevated temperatures and the relative stability of different octadecylsiloxane-bonded sorbents probably depends on the ability of the bonded phase to shield the silica substrate from dissolution in superheated water. The vapor pressure of water is modest, even at high temperatures, and by 200°C only reaches about 15 bar. Consequently, only moderate pressures are required to maintain a liquid state. A fixed restrictor or backpressure regulator set at 50 atm is usually sufficient to maintain superheated water in the liquid state throughout the temperature range for separations.

Superheated water provides access to a wider range of detection options for liquid chromatography compared with aqueous-organic mobile phases. Superheated water shows no absorbance down to 190 nm and the modest backpressure required to maintain the mobile phase in the liquid state allows the use of standard UV (and fluorescence) detectors. The signal to noise ratio can be improved by attaching cooling fins to the inlet capillary of UV detectors to act as a heat sink to minimize temperature-dependent refractive index changes in the flow cell. Packed capillary columns or conventional packed columns with a postcolumn splitter are simply interfaced to the flame ionization detector using a restrictor inserted into the detector base [235,236,241,242]. Liquid flow rates of about 100 μ l / min are accepted by the detector when the detector gas flow rates, detector temperature, and position of the restrictor tip with respect to the flame jet are optimized. Detector noise is about an order of magnitude higher than observed in gas chromatography resulting in proportionally higher detection limits. Superheated deuterium oxide is a suitable mobile phase for continuous or stopped flow detection by

nuclear magnetic resonance spectroscopy (LC-NMR) [243,244]. The absence of intense mobile-phase proton signals simplifies NMR experiments.

Reversed-phase separations using superheated water have not found any major applications so far, but a wide range of low molecular mass polar compounds have been separated at modest temperatures with relatively simple and rugged instrumentation. It has potential for wider use in laboratories that wish to reduce consumption of organic solvents. In addition, superheated water should be compatible with on-column preconcentration techniques for trace analysis

7.7.3 Solvating Gas Chromatography

In solvating gas chromatography, a supercritical fluid or superheated liquid is pumped into a packed capillary column with its exit at atmospheric pressure [241,245-248]. Along the column a transition occurs between the highly solvating fluid or liquid at the inlet and the gas phase exiting the column. Separations are achieved at a constant inlet pressure and column temperature, or by programming either the inlet pressure and column temperature or both simultaneously. Instrumentation is similar to that used for packed capillary column supercritical fluid chromatography except that the column outlet restrictor is no longer required.

The changing conditions along the column are difficult to model in any certain manner. However, neither the large change in mobile phase linear velocity nor the phase changes from superheated liquid to gas along the column seem to have a significant effect on column efficiency [247]. Solvating gas chromatography is more suitable for fast separations than conventional supercritical fluid chromatography or superheated liquid chromatography as long as the sample is eluted from the column [249]. Increasing the particle size of the column packing allows higher molecular mass compounds to be eluted under the same separation conditions. For simple mixtures containing weakly retained compounds solvating gas chromatography is superior to open tubular column gas chromatography for fast separations, since it allows favorable optimization of retention and selectivity factors [250]. For complex mixtures containing adequately retained compounds, however, open tubular column gas chromatography is the better choice. A large number of separations have been demonstrated using solvating gas chromatography, but if this method is to become more than a curiosity, it is necessary to demonstrate its suitability for applications that neither gas nor supercritical fluid chromatography can perform in an acceptable manner.

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8.1 INTRODUCTION

Capillary-electromigration separation techniques are a family of separation methods carried out in empty, coated or packed capillary columns with electrolyte solutions as the mobile phase. An electric field is responsible for driving the sample and mobile phase through the column by processes dependent on electrophoresis and electroosmosis. This common arrangement allows a similar instrument platform to service all capillary-electromigration separation techniques with only minor modifications for specific applications. These methods only recently entered analytical laboratories, although in a planar format they have a long history of use in biochemical and clinical laboratories [1-5].

From the beginning of free solution electrophoresis, band broadening from thermal effects was foreseen as the main problem restricting its development [6]. The energy generated as heat by application of an electric field to an electrolyte solution in a column raises the solution temperature, but more critically, results in a radial

temperature gradient between the column center and the wall. Convective transport between neighboring zones of different temperature emerges as a major contributing factor to band broadening, restricting the field strength that can be applied to improve resolution and minimize the separation time. A major development in the late 1940s and early 1950s was the introduction of anti-convective media in a planar format, such as paper and gels, which to some extent minimized thermal effects. In the case of gels, they also provided for the possibility of size separations by molecular sieving [1-5]. In many ways, a superior solution to thermal effects was the introduction of capillary columns, as a replacement for thin-layer gels, in the 1980s [7]. The high surface area to volume ratio of these columns was successful in minimizing radial temperature gradients, and allowed high field strengths to be used for fast separations. By the end of the 1980s, commercial instruments for capillary-electromigration separation techniques had appeared, and numerous research groups entered the field. During the 1990s, these techniques represented the most active research area in separation science [8-13]. By the end of the 1990s, capillary-electromigration separation techniques had reached a reasonable level of maturity with continuing interest driven by applications, accompanied by a slower evolution of instrumentation and theory.

In this chapter, we will discuss capillary-electromigration separation techniques exclusively because of their close relationship to chromatography, and the general presence of capillary electrophoretic instruments in analytical laboratories. The reader should note that this represents a distortion of the general use of electrophoretic techniques in science. Thin-layer gel electrophoresis continues to be widely used in biochemical and clinical laboratories for the separation of macromolecules (e.g. proteins, DNA fragments, etc.) [1-5]. Capillary-electromigration separation techniques are also used for these applications but, in addition, have found wide acceptance for the separation of small molecules as a complement to column liquid chromatography. Compared with capillary-electromigration separation techniques, the main disadvantages of thin-layer gel electrophoresis are a lack of complete automation, time-consuming and labor-intensive methods, slow separations, and mostly qualitative detection procedures [2,3,13,14]. Since capillary-electromigration separation techniques have not displaced thin-layer gel methods from laboratory use, the other side of the coin is that thin-layer gel methods are low cost, standardized, understood by laboratory workers, provide superior resolution in the two-dimensional format, and have good sensitivity [1,2,5]. Although complete automation of thin-layer gel methods is unavailable, advances in automation of gel casting, sample application, image detection, data analysis and interfaces to mass spectrometry are an indication that thin-layer gel technology is continuing to evolve. Thus, developments in thin-layer gel electrophoresis and capillary-electromigration separation techniques are occurring in parallel, and the premature obituary of thin-layer gel electrophoresis is unjustified from the perspective of contemporary laboratory use.

Capillary-electromigration separation techniques are said to reduce method development time, to reduce operating costs and solvent consumption, provide higher efficiency, and are easier to interface to some types of detectors compared with column liquid chro-

matography [11,15-17]. Their main disadvantages compared with column liquid chromatography are instrument related: such as poor injection precision and concentration sensitivity; poor retention reproducibility; and matrix interference on separation quality. Liquid chromatography is a mature technique with excellent commercial support. It is difficult to persuade industrial laboratories to convert from familiar methods to new technologies if the advantages of doing so seem relatively minor, and result in additional costs for training and method validation. The situation should not be viewed as entirely negative, since capillary-electromigration separation techniques do compete effectively with column liquid chromatography for some applications and have developed several specific applications of their own [2,11,12,15-19]. This has ensured the acceptance of capillary-electromigration separation techniques, even if commercial success judged by the relative size of the instrument market, is just a fraction of the market for gas and liquid chromatographic instruments.

Classification of capillary-electromigration separation techniques into elementary processes based on column chemistry and common applications allows a convenient division of the subject matter. Some confusion exists over general nomenclature, however, which is not standardized [2,10,20]. Capillary electrophoresis (CE) is sometimes used as a general description for the family of capillary-electromigration separation techniques, or specifically to describe separations by capillary zone electrophoresis (CZE). Capillary electrophoresis is used here rather than capillary zone electrophoresis to describe the separation of ions in an electric field based solely on differences in electrophoretic mobility in electrolyte solutions (section 8.2). It is the most straightforward of the capillary-electromigration separation techniques, and widely used for the separation of inorganic and organic ions, ionizable compounds, zwitterions, and biopolymers. Micellar electrokinetic chromatography (MEKC), sometimes called micellar electrokinetic capillary chromatography (MECC), is used to describe separations based on a combination of electrophoresis and two-phase distribution of analytes between the electrolyte solution and a secondary (or pseudostationary) micellar phase (section 8.3). For useful separations the migration velocity of the charged micelles must be different to the bulk electrolyte solution. Micellar electrokinetic chromatography is commonly used for the separation of water-soluble neutral compounds and weak acids and bases. Microemulsion electrokinetic chromatography (MEEKC or MEECC) is similar in most respects to MEKC, except that a microemulsion is used in place of the simple micellar secondary phase (section 8.3.5). Capillary electrochromatography (CEC) employs a packed or wall-coated capillary column through which the mobile phase is driven by electroosmosis, possibly assisted by pressure (section 8.4). Retention differences result from a combination of electrophoretic migration and chromatographic distribution mechanisms. Capillary sieving electrophoresis (CSE) is a general term used to describe ion separations based on size or shape differences, resulting from migration through a capillary column filled with a gel matrix (or solution of entangled polymer), to create a medium with an apparent range of pore sizes. Although approaches are different (gel *versus* entangled polymer solution), the separation mechanisms are similar, and for convenience I have lumped them together as capillary gel electrophoresis (CGE),

conforming to general practice (section 8.5). CGE is used primarily for the separation of macromolecules (e.g. DNA fragments and proteins). Capillary isoelectric focusing (CIEF) is an electrophoretic technique used for the separation of amphoteric analytes in order of their isoelectric points in a pH gradient formed in a capillary tube (section 8.6). CIEF is used primarily for the separation of proteins and other zwitterionic compounds. Capillary isotachopheresis (CITP) is an electrophoretic technique in which ions are separated in order of their electrophoretic mobility in a discontinuous buffer system. The sample ions form a chain of adjacent zones moving with equal velocity between two electrolyte solutions containing ions of greater (leading) and lower (trailing) mobility (section 8.7). CITP is mainly used as a preconcentration technique for ions and as the first separation dimension in some coupled column systems.

8.2 CAPILLARY ELECTROPHORESIS

Superficially separations by chromatography and electrophoresis look alike consisting of a series of (ideally) symmetrical peaks separated in time. Several descriptive terms borrowed from chromatography (e.g. selectivity, efficiency and resolution) are easily calculated from peak positions and shapes in an electropherogram providing a reasonable starting point for a general discussion of the theoretical aspects of electrophoresis. That being said, we should not lose sight of the fact that electrophoresis and chromatography are based on different separation principles with too great an emphasis on their similarities, a potential source of confusion. The full range of experimental variables and their interactions are different for chromatography and electrophoresis. Even if common terms, such as column plate count and resolution, can be determined in the same way, their connection to theoretical principles have little in common. As an alternative approach, a number of authors have described continuity models to describe electrophoretic separations [21-24]. In this case, the column is divided into a series of segments and equations set up for movement of ions by electrophoresis, electroosmosis and diffusion in each segment. Ion separations are simulated for a defined column length by summation of migration and dispersion contributions for the required number of segments. These models are complex, computationally intensive, and require exact or approximate numerical equations for all experimental and analyte physicochemical and system properties, which are not always available. Their advantage is that they allow optimization of separations for varied conditions by simulation.

8.2.1 Electrophoretic Mobility

Application of an electric field to an ion in solution causes the ion to move with a constant velocity that depends on the field strength, temperature and characteristic properties of the ion and electrolyte solution. The electrostatic force accelerating the ion ($F = qE$) is opposed by viscous forces in the solution restricting its movement. For a spherical particle the viscous force is given by Stokes' law ($F = 6\pi\eta r v_{ep}$). After a short

induction period, steady state conditions are achieved, and the electrophoretic velocity of the ion relative to the electrolyte solution, v_{ep} , is given by Eq. (8.1)

$$v_{ep} = qE / 6\pi\eta r_s \quad (8.1)$$

where q is the charge on the ion (the product of the number of elementary charges and the charge on an electron), E the electric field strength (V / L), V the voltage difference across the column, L the column length, η the solution viscosity, and r_s the hydrodynamic radius of the ion [12,13,25-30]. The hydrodynamic ion radius is expected to be larger than the crystallographic ion radius and includes contributions from the solvation shell.

For a simpler comparison of experimental data the electrophoretic mobility, μ_{ep} , defined as the analyte electrophoretic velocity at unit field strength, is generally used.

$$\mu_{ep} = v_{ep} / E \quad (8.2)$$

Ions can be separated when they differ in their charge-to-size ratio (q / r_s) corresponding to different electrophoretic mobility values. The electrophoretic mobility is independent of the field strength but depends on temperature (because this affects the solution viscosity [31]) and the composition of the electrolyte solution. Eq. (8.1) is only valid for spherical particles in dilute solutions with an ionic strength close to zero. For an electrolyte solution of finite concentration, ion-ion interactions reduce the mobility of sample ions relative to the limiting case of infinite dilution [32-34]. A diffuse cloud of oppositely charged electrolyte ions surrounds each sample ion. In an external electric field, this diffuse ion cloud will migrate in the opposite direction to the sample ion and exerts an additional force opposing the motion of the sample ion, referred to as the electrophoretic or retardation effect. In addition, restoration of the ion cloud around the moving sample ion requires a finite time, referred to as the relaxation time. The net result is that the ionic atmosphere becomes distorted and lags behind the moving sample ion. Since the ionic cloud and sample ion are of opposite charge, the movement of the sample ion is opposed by electrostatic interactions with the asymmetrical charge distribution of the ion cloud under electrophoretic conditions. Both the retardation effect and relaxation effect oppose the migration of the ion through the electrolyte solution, and result in a decrease in the electrophoretic mobility that depends on the ionic strength of the electrolyte solution. For typical electrolyte solutions used in capillary electrophoresis, the effect of ionic strength on the electrophoretic mobility can be expressed by

$$\mu_{ep} = \mu_0 \exp[-0.77 (zI)^{1/2}] \quad (8.3)$$

where μ_0 is the absolute electrophoretic mobility of the ion in pure water, z the charge number for the ion and I the ionic strength of the electrolyte solution [25]. Eq. (8.3) is useful for predicting the effect of ionic strength on ion mobility for electrolyte concentrations of 1-100 mM and $z = 1-6$.

Classical electrolyte theories were developed to explain equivalent conductance of electrolyte solutions and not mobility of sample ions at infinite dilution. In essence, such theories describe the electrophoretic behavior of the electrolyte and not the sample ions. Theories for the mobility of sample ions are difficult to formulate and are only poorly developed at present. A simple extension of classical electrolyte theories to electrophoretic mobility for ions of finite size, allows the derivation of Eq. (8.4) [32]

$$\mu_{ep} = \mu_0 - [(AzI^{1/2}) / (1 + 2.4I^{1/2})] \quad (8.4)$$

where A is an experimental constant (related to the Onsager slope) from plots of the electrophoretic mobility as a function of the ionic strength of the electrolyte. Eq. (8.4) has a similar application range as Eq. (8.3) but is not necessarily better than Eq. (8.3). For small organic molecules and inorganic ions at room temperature, μ_0 has values between $20\text{--}80 \times 10^{-9} \text{ m}^2/\text{Vs}$. By convention, ionic mobilities are signed quantities. Cations have positive mobilities and anions negative mobilities.

Under typical experimental conditions the observed electrophoretic velocity results from the combined effects of electrophoretic and electroosmotic migration. The origin and properties of electroosmotic flow are discussed in section 8.2.2. The experimentally observed electrophoretic velocity is given by Eqs. (8.5) and (8.6)

$$v_{ap} = v_{eo} + v_{ep} \quad (8.5)$$

$$\mu_{ap} = \mu_{eo} + \mu_{ep} \quad (8.6)$$

where the subscript ap indicates the apparent (also known as the observed or total) electrophoretic velocity (or mobility) and eo indicates the electroosmotic velocity (or mobility). The apparent and electrophoretic velocities (or mobilities) are characteristic properties of the ion while the electroosmotic velocity (or mobility) is a system property. The electrophoretic and electroosmotic velocities (or mobilities) are vector quantities and are summed with respect to their signs.

The effective mobility, μ_{eff} , is used to describe the overall electrophoretic mobility of the ionic forms of a compound or complex resulting from any number of equilibrated species, where equilibration is fast compared with the separation time [35–38]. In such cases, a single peak is observed for all interconverting species with an effective mobility that depends on the equilibrium constants for each species and the properties of the electrolyte solution. For example, Figure 8.1 illustrates a typical plot of the effective mobility of a monovalent weak acid as a function of the pH of the electrolyte solution. At low pH the effective mobility of the acid is zero. All forms of the acid are present in the neutral form and migrate along the column at the electroosmotic velocity. At higher pH the effective mobility of the acid exhibits the characteristic sigmoidal shape of the acid dissociation equilibrium as a function of pH. The effective ion mobility at any pH is proportional to the equilibrium mole fraction of the acid in the anionic form. At a sufficiently high pH, all of the acid is dissociated, and the effective mobility is

pK_a by Capillary Electrophoresis

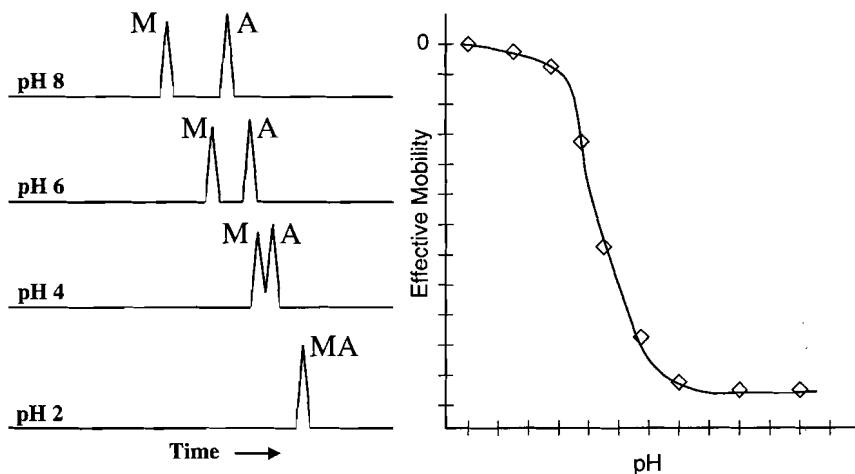


Figure 8.1. Influence of buffer pH on the effective electrophoretic mobility of a monovalent weak acid in capillary electrophoresis. The separations show the relative position of the anion (A) and a neutral compound (M), used as an electroosmotic flow marker.

identical to the electrophoretic mobility of the anion. Thus for a monovalent weak acid the effective mobility is given by

$$\mu_{\text{eff}} = \alpha \mu_{\text{ep}} \quad (8.7)$$

where α is the fraction of the monovalent acid present as the anion

$$\alpha = K_a / ([H^+] + K_a) = 1 / [1 + 10^{(pK_a - pH)}] \quad (8.8)$$

There is no restriction on the number of complexation or dissociation equilibria involved, and for the general case, the effective mobility is given by

$$\mu_{\text{eff}} = \sum_i x_i \mu_{\text{ep},i} \quad (8.9)$$

where x_i is the mole fraction of species i with an electrophoretic mobility $\mu_{\text{ep},i}$. Some examples of Eq. (8.9) for the calculation of the effective mobility of weak acids and bases are summarized in Table 8.1 [36-45]. Measurement of the effective mobility of a weak acid or base in a series of pH buffers at constant ionic strength affords a convenient method for determination of pK_a values when speed, a small sample size, and tolerance of impurities are important [46,47]. The thermodynamic pK_a values are obtained by conventional corrections for ionic strength and activity.

Table 8.1

Relationships used to calculate the effective electrophoretic mobility of weak acids and bases

Monoprotic acids and bases (with activity correction)

$$pK_a = pH - \log [\mu_{eff} / (\mu_{ep} - \mu_{eff})] + [0.509z^2]^{1/2} / (1 + 0.328a^{1/2}) \quad (\text{acids})$$

$$pK_a = pH + \log [\mu_{eff} / (\mu_{ep} - \mu_{eff})] - [0.509z^2]^{1/2} / (1 + 0.328a^{1/2}) \quad (\text{bases})$$

μ_{ep} = electrophoretic mobility of the fully ionized or protonated ion, I = ionic strength of the buffer, z = valance charge of the ion ($z = 1$ for monoprotic acids and bases) and a = ionic size parameter (for small molecules assumed to be 5 Angstroms). For a buffer with $I = 50$ mM at 25°C and $z = 1$ the correction term is ≈ 0.08 . For the weaker pK_a of a dibasic compound ($z = 2$) the correction term is ≈ 0.25 for the same conditions.

Monoprotic acids and bases

$$\mu_{eff} = (\mu_{ep} K_a) / (K_a + [H^+]) \text{ or } \mu_{eff} = [\mu_{ep} 10^{(pH-pK_a)}] / [1 + 10^{(pH-pK_a)}] \quad (\text{acids})$$

$$\mu_{eff} = (\mu_{ep} [H^+]) / (K_a + [H^+]) \text{ or } \mu_{eff} = \mu_{ep} / [1 + 10^{(pH-pK_a)}] \quad (\text{bases})$$

Zwitterionic monoprotic acid and base

$$\mu_{eff} = (\mu_{ep1} [H^+]^2 + \mu_{ep2} K_{a1} K_{a2}) / ([H^+]^2 + K_{a1} [H^+] + K_{a1} K_{a2})$$

$$\mu_{eff} = (\mu_{ep1} [10^{-pH}]^2 + \mu_{ep2} [10^{-(pK_{a1}+pK_{a2})}]) / ([10^{-pH}]^2 + [10^{-(pH+pK_{a1})}] + [10^{-(pK_{a1}+pK_{a2})}])$$

subscripts 1 and 2 refer to the acid and base groups, respectively

Diprotic acid

$$\mu_{eff} = (\mu_{ep1} K_{a1} [H^+] + \mu_{ep2} K_{a1} K_{a2}) / ([H^+]^2 + K_{a1} [H^+] + K_{a1} K_{a2})$$

$$\mu_{eff} = (\mu_{ep1} [10^{-(pH+pK_{a1})}] + \mu_{ep2} [10^{-(pK_{a1}+pK_{a2})}]) / ([10^{-pH}]^2 + [10^{-(pH+pK_{a1})}] + [10^{-(pK_{a1}+pK_{a2})}])$$

subscripts 1 and 2 refer to the dissociation of the first and second acid proton, respectively

Diprotic base

$$\mu_{eff} = (\mu_{ep1} [H^+]^2 + \mu_{ep2} K_{a1} [H^+]) / ([H^+]^2 + K_{a1} [H^+] + K_{a1} K_{a2})$$

$$\mu_{eff} = (\mu_{ep1} [10^{-pH}]^2 + \mu_{ep2} [10^{-(pH+pK_{a1})}]) / ([10^{-pH}]^2 + [10^{-(pH+pK_{a1})}] + [10^{-(pK_{a1}+pK_{a2})}])$$

subscripts 1 and 2 refer to the dissociation of the first and second proton, respectively

For weak acids and bases, the effective electrophoretic mobility is strongly influenced by environmental factors that affect the underlying equilibrium constants, such as pH, ionic strength, organic solvent, and temperature, as well as variables that affect the ion mobility, such as ionic strength, temperature and viscosity [34,48-53]. The effective mobility of most ionizable compounds is higher in aqueous electrolytes than in aqueous-organic solvent mixtures. Addition of an organic solvent to an aqueous electrolyte results in an increase in the pK_a of the analyte, corresponding to a reduction in the extent of ionization. In addition, the trend in the change of effective electrophoretic mobility with composition tends to reflect the change in viscosity for the mixture with a minimum in the effective electrophoretic mobility corresponding roughly with the maximum in the solution viscosity. Walden's rule, however, that the product of $\eta\mu_{eff}$ is a constant, is an oversimplification and only obeyed approximately.

Other examples of fast equilibria exploited for either separations or determination of physicochemical properties include metal-ligand complexation [36,54], formation of inclusion complexes with cyclodextrins [55] or crown ethers [54,56,57], ion-pair formation [58,59], and weak noncovalent complexes between small molecules and

biopolymers (affinity CE) [35,60-62]. The ligand (or complex-forming reagent in the case of cyclodextrins or crown ethers) is added to the electrolyte solution at a fixed concentration. The sample is injected into the separation system with formation of sample-ligand complexes in the electrolyte solution. If 1:1 association complex are formed, the effective mobility of the analyte is given by

$$\mu_{\text{eff}} = (\mu_{\text{ep}} + \mu_{\text{c}}K[L]) / (1 + K[L]) \quad (8.10)$$

where μ_{ep} is the electrophoretic mobility of the fully ionized analyte, μ_{c} the electrophoretic mobility of the complexed form of the analyte, K the equilibrium constant for complex formation and L the ligand (or complexing reagent) concentration. To determine equilibrium constants μ_{eff} at a constant analyte concentration is measured as a function of the ligand concentration and Eq. (8.10), in one of several possible linear transformations, is used for the calculation [35,60-64]. Meaningful measurements require that complex formation results in a reasonable change in the electrophoretic mobility of the analyte ($\mu_{\text{ep}} \neq \mu_{\text{c}}$) and that a careful selection of the linear transformation method in conjunction with weighted least squares regression is made. Mobility corrections for changes in ionic strength and solution viscosity are required in some cases to minimize errors. And, of course, unless activity corrections are made, the equilibrium constants cannot be considered true thermodynamic constants.

Many metal cations have identical charge and similar ionic radii. Consequently, differences in their electrophoretic mobility are often insufficient for adequate separation in the absence of a change in their charge-to-size ratio achieved through complexation. Due to the fast equilibrium between different complexes, it is possible to describe the system as a single complex with the average number of ligands (equivalent to the average degree of complexation), n_{av} , calculated as

$$n_{\text{av}} = (\beta_1[L^-] + 2\beta_2[L^-]^2 + \dots + m\beta_m[L^-]^m) / (1 + \beta_1[L^-] + 2\beta_2[L^-]^2 + \dots + m\beta_m[L^-]^m) \quad (8.11)$$

Ion separations result from differences in their average degree of complexation (n_{av}), which depends on the stability constants of the metal ions with the selected ligand (β). Eq. (8.11) provides an alternative method of calculation to Eq. (8.10) in an extended form to include the correct order for the metal-ligand complex ratio [36,37,54]. This approach is also applicable to analyte-cyclodextrin complexes containing more than one cyclodextrin group [65]. Enhanced selectivity in the case of cyclodextrins and crown ethers is attributed to their ability to selectively include a wide variety of organic and inorganic ions into their hydrophobic cavity. When the solute forms a complex with the cyclodextrin or crown ether, its electrophoretic mobility is significantly reduced owing to the change in apparent size. Charged cyclodextrins have been used for the separation of neutral analytes capable of forming inclusion complexes affording separations similar to those obtained in micellar electrokinetic chromatography.

In the absence of electroosmotic flow, the electrophoretic mobility of an ion is calculated from the electropherogram by

$$\mu_{ep} = (L_D / V t_m) \quad (8.12)$$

where L_D is the distance from the injection end of the capillary to the detector and for on-column detection will be shorter than the column length L over which the voltage V is applied, and t_m is the migration time of the ion. In the presence of electroosmotic flow the electrophoretic mobility is calculated by

$$\mu_{ep} = (L_D / V) [(1/t_m) - (1/t_{eo})] \quad (8.13)$$

where t_{eo} is the migration time of a neutral marker compound that is carried through the column by the electroosmotic flow (section 8.2.2). Substitution of the appropriate migration times into either Eq. (8.12) or (8.13) allows calculation of the electroosmotic mobility (μ_{eo}), the apparent (total or observed) electrophoretic mobility (μ_{ap}) and the effective electrophoretic mobility (μ_{eff}). Some authors prefer to use the symbol m for mobility in place of μ for any of the defined electrophoretic or electroosmotic mobilities with retention of the appropriate subscript.

For computer simulation of electrophoretic separations, it would be useful to have calculation methods that allow a reliable estimate of electrophoretic mobility from structure and system properties [13,28,29,66]. All contemporary models in one way or another, are derived from Stokes' law, and are only strictly valid for the motion of spherical ions in solutions of low ionic strength. It seems generally true that various descriptors related to the ion volume can account for a large portion of the change in electrophoretic mobility for ions with the same charge but fail to account for differences in mobility related to shape. Chemical structure has a decisive influence on mobility, as indicated by the facile separation of many types of positional isomers that have the same charge. To better account for shape, additional terms to the effective size (usually characterized by $1/r_{vdw}$ where r_{vdw} is the van der Waals radius) are added to regression models to account for orientation in the applied electric field. For example, addition of a rotational radius and an inertial radius were used to improve the fit to experimental electrophoretic mobilities for alkylpyridines [28]. The different radii were calculated by molecular modeling techniques. For simple ions, it is generally observed that the mobility of multiply charged ions is less than z times that of a singly charged ion of similar size (where z is the formal charge). A number of semi-empirical relationships have been proposed to calculate the mobility of peptides [26,30,67-69]. Most models estimate the charge from the ionization constants of the amino acids, and relate the Stokes' radius to the molar mass (M) or number of amino acid residues. In general, the various models suggest a direct dependence of the electrophoretic mobility on the effective charge and a size dependency described by $(1 / M^a)$, where a has values between 1 and 1/3. The effective charge on the peptide is invariably calculated from the Henderson-Hasselbalch equation, assuming a standard set of pK_a values for each

amino acid, and ignoring electrostatic and steric interactions between neighboring residues. None of these two parameter models, however, can predict mobilities with sufficient accuracy to determine migration order and peptide positions in complex electropherograms.

8.2.2 Electroosmotic Flow

Electroosmotic flow is the motion of an electrolyte solution under the influence of an electric field that results from the charge imbalance at a solid-liquid interface [13,70-74]. It supplements the electrophoretic motion of ions in capillary electrophoresis. Electroosmosis provides the general transport mechanism for neutral compounds in electrophoretic experiments, as well as contributing to the apparent electrophoretic mobility of ions (see Eq. 8.5 and 8.6). For typical experimental conditions, the electroosmotic velocity (or mobility) of the electrolyte solution is generally greater than the electrophoretic velocity (mobility) of the sample ions. This has several consequences, both favorable and undesirable. For a fused-silica capillary column, the direction of electroosmotic flow is towards the cathode (negative electrode). If the electroosmotic mobility is greater than the electrophoretic mobility of sample ions then both cations and anions can be determined by a single detector when injection is made at the anode end of the column and the detector is located upstream of the cathode. This is the usual set-up for general operation. Low electroosmotic flow is often favorable for the resolution of closely spaced peaks, while high electroosmotic flow favors fast separations. In general, however, it is more desirable to obtain a reproducible electroosmotic velocity, irrespective of its value, since variation in the electroosmotic flow between different runs and different columns is the main source of poor repeatability of migration times in capillary electrophoresis.

When an insulator is immersed in an electrolyte solution, an electrical double layer is formed at the interface. Adsorption of ions from solution, or dissociation of surface functional groups, is responsible for the formation of a charged surface. In the case of fused-silica capillary columns in contact with an electrolyte solution with a $\text{pH} > 2\text{-}3$, the surface possesses an intrinsic negative charge due to the presence of weakly acidic silanol groups. At the silica surface a slight excess of positive charges in solution exists to balance the fixed negative charges on the silica surface forming an electrical double layer, Figure 8.2. Some of these excess cations are firmly held in the region of the double layer closest to the capillary wall (the compact or Stern layer). These cations are believed to be less hydrated than those in the diffuse region of the double layer. The excess charge density associated with the free ions falls off rapidly with distance from the surface, along with the associated electrical potential, which is proportional to the charge density. The thickness of the electrical double layer is small, typically about 10 nm. The potential at the boundary between the charged surface and the start of the diffuse part of the double layer is called the zeta potential, denoted by ζ , and is typically between 0 and 100 mV. Application of an electric field causes the cations in the diffuse portion of the double layer to migrate towards the cathode. Strong

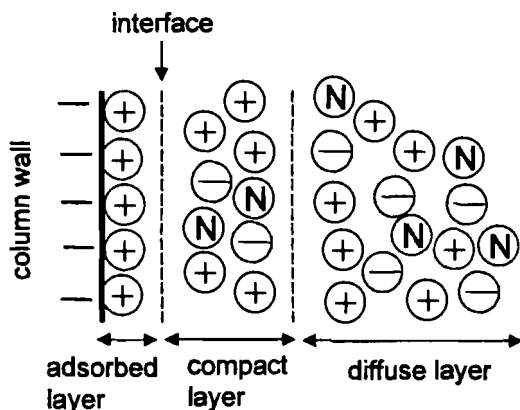


Figure 8.2. Representation of the electrical double layer at the surface of the inner wall of a fused-silica capillary column. (From ref. [72]; ©Elsevier).

dipole-type interactions between water molecules in the high electric field close to the capillary surface results in a local increase in the viscosity of the solution in the compact layer, which is immobile under typical operating conditions. But in the diffuse layer the migration of the cations and their associated hydration shell induces bulk flow of the whole solution within the capillary with a velocity profile that is virtually flat across the capillary diameter (except in the immediate vicinity of the capillary wall). General theory represents the electrolyte solution as a continuous dielectric that interacts electrostatically with the charged surface, and for a planar surface

$$v_{eo} = \epsilon_0 \epsilon \zeta E / \eta \quad (8.14)$$

where ϵ is the solution dielectric constant and ϵ_0 the permittivity in a vacuum. The observed electrophoretic velocity depends on the surface charge density on the inner surface of the capillary, temperature, and the ionic strength and viscosity of the electrolyte solution. For fused silica at pH < 3 the electroosmotic velocity is low and reaches a maximum at about pH 7-8. Plots of the electroosmotic mobility against pH are flatter than classical titration curves, reflecting the heterogeneity of the dissociation constants of the surface silanol groups. At higher ionic strength the larger concentration of cations in the compact layer diminishes the zeta potential at the shear plane and reduces the electroosmotic velocity. Addition of organic solvent to the electrolyte solution generally reduces the electroosmotic velocity by reducing the surface charge density and the dielectric constant of the electrolyte solution, as well as possibly increasing the solution viscosity.

Most approaches for controlling the electroosmotic velocity focus on altering the effective surface charge density of the capillary wall either by modification of the surface with a polymer layer (section 8.2.7.2), or by dynamic coating with additives

incorporated into the electrolyte solution (section 8.2.7.1). The types of additives commonly used include multivalent metal cations, alkylammonium ions, cationic polymers and surfactants. These additives can be broadly classified into: (1) those additives that ion exchange with surface silanol groups to reduce the surface potential; and (2) those additives that coat the capillary wall to shield silanol groups and possibly reverse the direction of the electroosmotic flow. Metal cations undergo an ion exchange type equilibrium with siloxide anions on the fused silica surface reducing the effective charge [71,75]. Multivalent cations like Mg^{2+} have a higher affinity for the charged surface and are more effective additives than monovalent cations, which provide only minor modification of the electroosmotic flow velocity. For the same reason diamines [e.g. 1,6-bis(trimethylammonium)hexane dibromide] are more effective additives for electroosmotic flow control than monoamines. The most popular additives for electroosmotic flow control, however, are cationic surfactants, such as cetyltrimethylammonium bromide or Fluorad FC 134 [72,76]. With increasing surfactant concentration the electroosmotic flow is first suppressed and eventually reversed in direction, finally reaching a plateau value above some critical surfactant concentration. The maximum value of the reversed flow is largely independent of pH and only slightly affected by the ionic strength of the electrolyte solution. It is more critically influenced by the solution counterion, being higher for singly charged inorganic ions and lower for organic and multivalent anions. The reversal of the electroosmotic flow by surfactants result from aggregation at the charged wall surface with formation of either a micellar layer or a bilayer. In effect, the wall surface charge becomes positive when the charge density of the adsorbed surfactant exceeds that of the ionized silanol groups. Reversed electroosmotic flow can be fine-tuned using mixtures of a zwitterionic surfactant and a cationic surfactant. The zwitterionic surfactant has a net charge of zero and in effect dilutes the positive charge at the capillary wall when mixed with the cationic surfactant.

Dynamic control of the electroosmotic flow velocity is possible by application of an external radial electric field to the fused-silica capillary [77-79]. This electric field is in addition to the longitudinal electric field applied to the capillary to cause electrophoretic migration. The radial electric field interacts directly with the surface charge that controls the zeta potential. Effective flow control for fused-silica capillaries is limited to a buffer pH < 5 due to the high surface charge density at high pH. The lower surface charge density of alkylsiloxane chemically bonded capillaries allows a greater range of electrolyte composition and pH to be used. Poor reproducibility, and other practical reasons, explain why this approach is little used in practice.

A number of methods are used to determine the electroosmotic flow velocity [80-84]. These include measuring the change in level or increase in mass of the receiving vial, monitoring the movement of a dye molecule in a capillary downstream of the detector, and on-line photometric detection of a neutral marker (such as mesityl oxide, formamide, acetone, thiourea, dimethyl sulfoxide, methanol, etc). The neutral marker method is straightforward and involves injection of a dilute solution of the marker compound in the separation buffer or water either separately or together with sample.

Due to prohibitively long migration times, however, this method is unsuitable for the measurement of low electroosmotic velocities in wall-modified capillary columns. In this case, an accurate value of the electroosmotic velocity is obtained by two or three separate and sequential injections of the marker solution with the separation voltage applied for a fixed time between consecutive injections. The sandwich of injection bands are then displaced by pressure from the injection end of the column and recorded by the detector. The time difference between the separately injected bands is used to calculate the electroosmotic velocity.

8.2.3 Band Broadening Mechanisms

The uniformity of the flow profile is of great importance for high separation efficiencies in capillary electrophoresis. Dispersion effects due to flow non-uniformity, as present in all forms of pressure-driven chromatography, are (ideally) absent in capillary electrophoresis. Thus the ultimate separation efficiency in capillary electrophoresis is determined only by longitudinal diffusion

$$N = (\mu_{ap}V / 2D)(L_D / L) \quad (8.15)$$

where N is the plate number for a particular analyte and D is the diffusion coefficient for the ion in the electrolyte solution [6,12,13,16,17,27,85]. The ultimate separation efficiency is independent of the column length. By using shorter columns, the separation speed can be increased without compromising the separation efficiency. Macromolecules have smaller diffusion coefficients, and therefore, exhibit higher separation efficiency compared with low-mass ions. The separation efficiency increases with the voltage used to drive the separation. The advantage of using narrow capillaries for separations with a high plate number is directly related to the higher voltages that can be applied owing to the efficient dissipation of heat by capillaries with a high surface area-to-volume ratio. The column length, internal diameter, and the electric field strength, therefore, are not independent variables, but are related through Joule heating of the electrolyte and its effect on the electrolyte flow profile. Taking these considerations into account Eq (8.15) predicts that plate numbers $> 500,000$ are possible for low-mass ions and > 1 million for macromolecules.

In practice, the separation efficiency is usually less than the ultimate separation efficiency due to secondary sources of band broadening, such as: extracolumn effects; Joule heating; wall adsorption; local turbulence due to non-uniformly charged capillary walls; hydrostatic flow; electromigration dispersion; and nonideal column geometry due to coiling or irregularities in shape [86-90]. A more general form of Eq. (8.15) can be written as

$$N = L_D^2 / (2Dt_m + \Sigma \sigma_i^2) \quad (8.16)$$

where t_m is the migration time of the ion and $\Sigma \sigma_i^2$ the sum of all variances describing independent sources of dispersion. Eq. (8.16) assumes a linear additive model, similar

to that proposed for chromatography (section 1.5.4). It simplifies the description of the band broadening process, and is partially justified for low sample concentrations in the electrolyte solution. Alternative theory that includes non-linear effects is more complex and requires individual analytical and numerical procedures for solution [89,91,92]. This approach is not considered here.

The use of narrow bore columns for efficient dissipation of Joule heat presents a problem owing to the finite dimensions of the injection zone and detection window associated with these systems [85,87,93]. Band broadening from non-ideal injection results from the introduction of the sample into the capillary by convection, migration or diffusion, and the loss of sample from the capillary by the same means after it is returned to the buffer. Processing of the detector signal is affected by the difference in size of the detection window or radiation beam, which ever is smaller, and the actual peak width of the sample ions. The spatial variance is identical for all sample ions but the temporal variance for each ion depends on the electrophoretic velocity of the ion, in contrast to chromatography, where all sample components elute from the column with a constant velocity [85]. Extracolumn contributions to band broadening are probably never zero for capillary electrophoresis instruments and increase in importance with a reduction of column dimensions. Since these contributions seem unavoidable, they have to be tolerated and minimized by good experimental practice. For typical operating conditions, instrumental contributions to band broadening are perhaps about 30% of the contribution from longitudinal diffusion, although this estimate is just a rough guide and cannot represent all possible experimental conditions. Approximate relationships for different instrumental contributions to band broadening are summarized in ref. [12].

During electrophoresis, the current passing through the electrolyte solution is responsible for the generation of heat [6,12,13,94]. Heat is produced homogeneously throughout the electrolyte solution resulting in an increase in temperature, but this is less important than the fact that heat loss occurs predominantly through the capillary wall, producing a radial temperature gradient. The temperature gradient is parabolic with a maximum at the capillary center. Since electrophoretic mobility increases at higher temperatures owing to a lower viscosity, ions in this viscosity gradient migrate at different velocities. The velocity dispersion is mitigated by radial diffusion, which enables ions to change velocity as they diffuse among different radial positions. This relaxation mechanism will be more effective for low-mass ions compared with macromolecules because of their higher diffusion coefficients. For a given system, the rate of heat production is directly proportional to the molar conductivity and concentration of the electrolyte solution, the electric field strength squared, and the column diameter squared. It is inversely proportional to the column length. Thus, heat production will be less for electrolyte solutions of low conductivity, such as zwitterionic buffers, and for low electrolyte concentrations, provided that a minimum concentration is maintained to minimize electromigration dispersion (section 8.2.5.1). The most effective means of minimizing radial temperature gradients is to reduce the column diameter to promote rapid heat transfer to the column wall. At typical field strengths, radial temperature gradients are generally insignificant for columns with diameters

below about 0.1 mm. Thus, although there is no direct relationship between the column diameter and column efficiency, for high efficiency small diameter columns should be employed to maintain a flat flow profile. The temperature rise also depends on heat removal from the outside of the capillary wall. Forced air convection, flowing liquid baths, or Peltier coolers are often employed with small diameter capillaries to improve the efficiency of heat removal and the reproducibility of migration times.

A number of experimental factors affect peak shapes, and therefore, the separation efficiency. Electromigration dispersion is discussed later (section 8.2.5.1). Wall adsorption invariably results in diminished separation performance, and depending on the adsorption rate constants, may provoke peak shape distortion or excessive retention, such that the results are unsatisfactory [91,92,95-97]. If wall retention occurs, radial mass transfer becomes necessary for equilibrium, and a large contribution to the plate height results. Several wall-modification procedures are employed to reduce the impact of wall-adsorption on the separation (section 8.2.7.2). When the electrolyte solution level in one electrode vial is higher than in the other, siphoning of liquid results in a flow of solution with a parabolic profile, at least for capillary columns with internal diameters > 0.05 mm. When this profile is superimposed on the flat electroosmotically-driven flow profile this results in additional band broadening caused by the radial flow velocity distribution. The flow profile can also be distorted by differences in the electroosmotic velocity over the length of the capillary. These flow inequalities may be caused by differences in the surface properties of the inner capillary wall, or from differences in solution pH over the length of the capillary, for example, when a large sample volume is injected.

For the most part, secondary contributions to band broadening are minimized by careful experimental design. Local turbulence and hydrostatic flow can be minimized by thoroughly conditioning the capillary and by equally positioning as well as filling the electrolyte reservoirs in which the ends of the separation capillary reside. To minimize contributions from injection, the injection plug length is kept as small as possible, and generally less than 1% of the effective column length. Electromigration dispersion and thermal effects are controlled by optimizing the conductivity difference between the sample and separation buffer and by operating at a voltage where heat is effectively dissipated. The highest voltage that can be used without the onset of thermal effects is just below the region where deviations from linearity occur in a plot of current against voltage.

8.2.4 Resolution

The operating definition of resolution in capillary electrophoresis is the same as that for chromatography (section 1.6.1). It is equal to the separation between two peaks divided by the average of their widths at base

$$R_S = \Delta t / 2(\sigma_{t,1} + \sigma_{t,2}) \quad (8.17)$$

where Δt is the difference in migration time for the two peaks ($t_{m2} - t_{m1}$) and σ_t the peak standard deviation in time units. To relate resolution to the experimental variables, the selectivity factor (analogous to the separation factor in chromatography) and the average plate number for the two peaks are introduced

$$R_S = 0.25[\Delta\mu_{ep} / (\mu_{av} + \mu_{eo})]N^{1/2} \quad (8.18)$$

where $\Delta\mu_{ep} / (\mu_{av} + \mu_{eo})$ is the selectivity, defined as the difference in the electrophoretic mobility for the two ions divided by the average of their average electrophoretic mobility and the electroosmotic mobility [98-101]. The electrophoretic mobility difference and the average electrophoretic mobility in Eq. (8.18) is replaced by effective mobilities and their average for ions affected by secondary chemical equilibria (section 8.2.1). Because there is no phase equilibrium involved in the separation, unlike chromatographic processes, there is no term containing the retention factor in Eq. (8.18). Since resolution increases with the square root of the plate number, and linearly with selectivity, the selectivity term in Eq. (8.18) is more influential for improving resolution. Eq. (8.18) can be rearranged into its operational form, with the assumption that longitudinal diffusion is the only factor contributing to efficiency and the ion diffusion coefficients can be represented by a single value

$$R_S = 0.25[\Delta\mu_{ep} / (\mu_{av} + \mu_{eo})^{1/2}][V L_D / 2DL]^{1/2} \quad (8.19)$$

Resolution is seen to increase with the square root of the applied voltage, up to the point where thermal effects become an important source of band broadening. Resolution reaches a maximum when $\mu_{eo} \approx -\mu_{av}$, however, the separation time will be approximately infinite. Experimental conditions, which approximate this equality, are only useful for the separation of ions with near identical electrophoretic mobilities (e.g. isotopes) [102]. In addition, since mobilities are signed quantities, it requires a larger difference in mobility to obtain a complete separation of ions migrating in the same direction as the electroosmotic flow than for ions migrating against it. In practice, optimization of resolution can be quite complex because of the number of parameters that affect efficiency and selectivity (electrolyte concentration, buffer composition, pH, voltage, temperature, type and concentration of organic solvent and concentration of any complexing or ion interaction reagent) and their interactions [36,43,48,54-58,99-101,103]. Changes in experimental variables often affect efficiency and selectivity simultaneously. Guidelines for selection and optimization of experimental variables are presented later (section 8.8).

8.2.5 Electrolyte Solutions

The purpose of the electrolyte solution (background electrolyte) is to provide stable conditions for the separation independent of sample concentration [8,13,104,105]. This requires that the field strength, electroosmotic flow and effective mobility of

Table 8.2

Capillary electrophoresis buffers

Mobility values are at zero ionic strength and 25°C (in 10^{-9} m² / Vs)

Buffer	pK _a	Mobility
Phosphoric acid	2.12 (pK ₁)	-35.1
	7.21 (pK ₂)	-58.3
	12.32 (pK ₃)	-71.5
Malonic acid	2.90 (pK ₁)	
	5.70 (pK ₂)	
Citric acid	3.13 (pK ₁)	-28.7
	4.76 (pK ₂)	-54.3
	6.40 (pK ₃)	-70.8
Trichloroacetic acid	2.35	-34.2
Lactic acid	3.85	-35.8
Hydroxyisobutyric acid	3.97	-33.5
Tetraboric acid	4.00	-30.0
Glutamic acid	4.38	-28.9
Acetic acid	4.76	-42.4
MES [2-(N-morpholine)ethanesulfonic acid]	6.13	-26.8
MOPS [3-(N-morpholine)propanesulfonic acid]	7.20	-24.4
MOPSO [2-hydroxy-4-morpholinepropanesulfonic acid]	6.79	-23.8
ACES [N-2-acetamido-2-aminoethanesulfonic acid]	6.84	-31.3
Imidazole	7.17	52.0
BES [2-(bis{2-hydroxyethyl}amino)ethanesulfonic acid]	7.16	-24.0
HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]	7.51	-21.8
TRICINE [N-(tris(hydroxymethyl)methyl)glycine]	8.15	
TRIS [tris(hydroxymethyl)aminoethane]	8.08	29.5
TAPS [3-(tris(hydroxymethyl)methyl)aminopropanesulfonic acid]	8.30	-25.0
BICINE [N,N-bis(2-hydroxyethyl)glycine]	8.35	
Glycylglycine	8.40	
Ammonia	9.26	
Ethanolamine	9.50	44.3
CHES [2-(cyclohexylamino)ethanesulfonic acid]	9.50	
Triethylamine	9.87	
CAPS [3-(cyclohexylamino)propanesulfonic acid]	10.40	
Diethylammonium	11.40	37.9

the sample ions remain constant throughout the separation. The usual control media are dilute aqueous solutions of pH buffers (nonaqueous capillary electrophoresis is discussed in section 8.2.6). General properties of aqueous buffers and their preparation are described elsewhere [104-108]. Desirable buffer properties for capillary electrophoresis include detector compatibility (usually non-UV absorbing), reasonable water solubility, an acceptable shelf life, and availability in a high purity form at a reasonable cost. The most popular buffers for capillary electrophoresis are phosphate, acetate, borate and zwitterionic buffers (Good's buffers), Table 8.2. Since most of Good's buffers complex metal ions, a secondary series of non-complexing zwitterionic buffers have been developed for applications where metal complex formation is undesirable [109].

Specific buffer properties of interest for capillary electrophoresis are its pH buffering range, capacity, specific conductivity, ion mobility and ionic strength [13,16,17,104,105,110,111]. Buffers are most effective within a 2-unit pH range bracketing the pK_a of the acid ($pK_a \pm 1$). The pH of the buffer affects the ionic state of the capillary wall and the effective charge of weak acids and bases. Both electrophoretic mobilities and electroosmotic mobility (section 8.2.1) as well as sample-wall interactions are affected. The buffer capacity must be sufficient to ensure that the local pH and conductivity are unchanged by the presence of the sample. The ionic strength of the electrolyte solution has a significant effect on mobility and selectivity for ions that differ in charge number. The specific conductivity of the buffer and the applied field strength control the heat generated in solution leading to changes in ion mobilities and possibly reduced separation efficiency by formation of radial temperature gradients (section 8.2.3). Buffers of low specific conductivity are generally preferred. Addition of organic solvents to electrolyte solution allows the separation of ions with low water solubility, modifies the acid-base equilibrium constant of weak acids and bases, reduces the electroosmotic mobility, may result in an increase in the solution viscosity, and usually reduces the solution dielectric constant.

Buffer properties can change during a separation because of electrochemical reactions at the electrodes [112,113]. The most general reaction is the hydrolysis of water, which leads to an accumulation of hydrogen ions at the cathode and their depletion (increase in the concentration of hydroxyl ions) at the anode. Changes in pH values in the electrode reservoirs depend on the buffer capacity, separation time, applied voltage, capillary cross-sectional area, and electrode reservoir volumes. The rate of pH change is greater for buffers with high mobility ions. The electrical conductivity and ionic strength of the buffer also change, affecting reproducibility of consecutive separations performed with the same buffer reservoirs. Frequent buffer replenishment is generally recommended.

8.2.5.1 Electromigration Dispersion

Electromigration dispersion is a peak deformation mechanism that occurs when the sample solution has a higher conductivity than the background electrolyte, and during separations where the migration velocity of the sample and buffer ions (or other ions) of the same charge are different [13,88,114,115]. Electromigration dispersion mostly results in the formation of triangular peak shapes and can significantly deteriorate the separation efficiency. This non-linear phenomenon is the result of distortion of the electric field in the sample zone by the presence of sample ions.

High sample-ion concentrations in the background electrolyte solution impose conductivity changes on the sample zones accompanied by changes in the local field strength. If the conductivity of the sample zone is increased by the sample ion concentration, the electrical field strength (and therefore the electrophoretic migration velocity) will be lowered in those parts of the zone with a high sample ion concentration. A zone with an originally symmetrical concentration profile will be deformed, since the zone front and tail will migrate with a different velocity compared with the peak

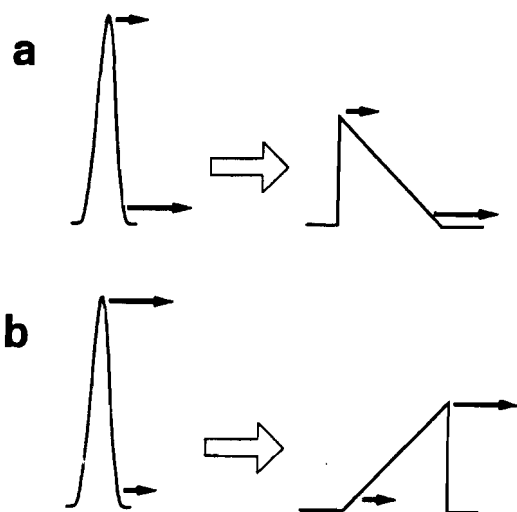


Figure 8.3. Band broadening and peak deformation when the migration velocity decreases (a) or increases (b) with sample concentration. (From ref. [13]; ©Vieweg Publishing).

apex. The result is a more or less triangular-shaped zone with a steep front on one side and a ramp on the other side, Figure 8.3. The zone shape and the extent of deformation depend on the sample ion concentration, the conductivity difference for the sample ions and electrolyte solution, and the peak migration time. For weak acids and bases peak deformation can result from changes in pH across the zone in the direction of migration induced by changes in the effective mobility of the sample ions. To minimize electromigration dispersion the sample ion concentration should be at least two orders of magnitude below the concentration of the background electrolyte, sample detectability permitting. Alternatively, the ionic strength of the background electrolyte can be increased within the limits set by Joule heating.

Any mismatch in mobility of sample ions and electrolyte co-ions will render the local electrical field strength different to that of the buffer. If the migration velocity of an individual sample ion exceeds that of the electrolyte co-ions, the local electric field will be lower for the sample zone with respect to the background electrolyte. Thus, any sample ions diffusing out of the zone will experience a higher electric field increasing their migration velocity. This causes the ions at the rear of the sample zone to re-enter the zone, whereas ions at the front of the zone will drift away from the zone. The overall effect is the formation of a tailing band with a sharp rear boundary and a broadened front. Similarly, if the sample ion has a lower mobility than the co-ion, a fronting band is expected. This form of peak deformation can be avoided by matching the mobility of sample ions to those of the electrolyte co-ions. For the average case, however, sample ions will have a wide range of mobility, and therefore, for most of the zones some zone broadening will be unavoidable.

8.2.5.2 *System Peaks*

System peaks can be formed under usual operating conditions in capillary electrophoresis [114-119]. System peaks do not contain sample ions and are defined as zones containing electrolyte ions with a different composition to the electrolyte solution. If the mobility of a sample ion is close to the mobility of a system peak, there is a strong interaction between the sample ion and the system peak resulting in peak deformation and a local decrease in the separation efficiency. The number and mobility of the system peaks depends on the composition of the electrolyte solution. An electrolyte solution containing n ionic species (both anions and cations are counted), results in the formation of $n-1$ system peaks. Of these, one system peak is considered stationary, and effectively migrates as an electroosmotic flow marker. The system peak corresponding to the electroosmotic flow results from the introduction of the sample solvent and is often observed as a dip or peak at the start of the electropherogram. Other system peaks have a characteristic mobility, which can be calculated from theory, but the process is complicated [115,116,119]. For electrolytes at very high or low pH, the hydroxyl or hydrogen ions act as a second co-ion and should be included in the calculation of the number of system peaks. Multivalent electrolytes (e.g. phosphate, phthalate, etc.) behave as electrolytes containing multiple co-ions and give rise to additional system peaks. Indirect absorbance detection (section 8.9.5.3) requires addition of an absorbing ion to the electrolyte solution with the concomitant formation of additional system peaks. In this case the ratio of the various co-ion concentrations must be adjusted to move the system peaks out of the region of the electropherogram used for measurements.

8.2.6 *Nonaqueous Electrolyte Solutions*

Most separations by capillary electrophoresis employ aqueous electrolyte solutions. Early studies of nonaqueous electrolytes garnished little attention, and it is only in recent years that systematic studies and applications have started to appear on a regular basis [120-122]. Although some organic solvents have sufficient protolysis constants to be used without addition of electrolytes, in practice the addition of electrolytes is nearly always required to obtain reproducible and stable results. Nonaqueous solvents allow the use of higher electrolyte concentrations and higher electrical field strengths without generating excessive heat, and an increase of the sample load using capillaries of wider internal diameter [123,124]. Low heat production is a result of the low electrical conductivity of nonaqueous electrolyte solutions, reflecting the existence of fewer solvent-separated ions (i.e. more favorable electrostatic interactions between ions). Higher diffusion coefficients in nonaqueous solutions result in lower separation efficiency that depends on the electrolyte concentration and the temperature distribution inside the capillary [90,125]. The plate number decreases with increasing ionic strength, but is lower for water compared with organic solvents.

Organic solvents show a different solvating power relative to each other and to water, Table 8.3. The driving force for exploring nonaqueous electrolyte systems is that the right choice of solvent may yield separations that are impossible in

Table 8.3
Properties of solvents used in capillary electrophoresis

Solvent	Boiling point (°C)	Viscosity (cP)	Dielectric constant	pK _{auto}
Methanol	64.7	0.545	32.70	17.20
Ethanol	78.3	1.078	24.55	18.88
Propan-1-ol	97.2	1.956	20.33	19.43
Acetonitrile	81.6	0.341	37.5	≥33.3
Tetrahydrofuran	66	0.46	7.58	
Formamide	210.5	3.30	111.0	16.8
N-Methylformamide	180	1.65	182.4	10.74
N,N-Dimethylformamide	153	0.802	36.71	29.4
Water	100	0.89	78.39	14.0

aqueous systems. Although nonaqueous capillary electrophoresis allows for numerous solvents as potential candidates, separation is only possible if the solute is charged or interacting with a charged additive. Organic solvents provide increased solubility for compounds with low water solubility and have a significant effect on the acid-base properties of weak acids and bases [126,127]. Water and some solvents like formamide and N-methylformamide have high dielectric constants allowing only weak electrostatic interactions [128]. Most other organic solvents, however, have much lower dielectric constants allowing weak electrostatic interactions (e.g. charge transfer, ion pair formation) to be exploited more fully than in aqueous capillary electrophoresis [34,120,129]. For example, the complexation of polycyclic aromatic hydrocarbons with planar cations, the charge-transfer complexation of N-heterocyclic compounds with silver salts and hydrogen-bond association of poly(ethylene glycol) with organic anions have virtually no parallel in aqueous solution chemistry. On the other hand, additives commonly used in aqueous solution chemistry, such as surfactant-formed micelles or inclusion complexes with cyclodextrins, are generally ineffective in the absence of the characteristic hydrophobic properties associated with water.

Solvents with low dielectric constants are of limited practical use, since virtually no solvent separated ions exist, and most studies to date have used alcohols (particularly methanol), acetonitrile and their mixtures [128-131]. For alcohols, a mixture of 25 mM ammonium acetate and 1 % (v/v) acetic acid is a popular electrolyte (the acetic acid serving as a proton source for neutral bases). Other common electrolytes include perchloric acid and tetraalkylammonium perchlorate salts.

8.2.7 Column Coating Procedures

The silanol groups on the inner surface of fused-silica capillary columns are responsible for the production of electroosmotic flow and also act as sites for the adsorption of sample ions, particularly cations. Surface coatings are used to block these active sites resulting in a reduced electroosmotic flow, or even reversal of the flow direction, as well as mitigating sample-capillary wall interactions [72,133-134]. Sample-wall interactions are undesirable because they invariably lead to lower separation efficiency

and possibly distorted peak shapes and even reduced recovery of sample mass. The stability and pH-dependence of the electroosmotic flow affects the reproducibility of migration times, and its magnitude and direction affects resolution and the separation time. Capillary wall coating techniques are conveniently divided into dynamic and surface-bonding techniques. Dynamic coating methods rely on the physical adsorption of various kinds of additives from solution. Chemically bonded surface layers are formed by the covalent attachment of a polymer film or ligand to the capillary wall before the column is placed in service. Dynamically coated columns are generally easy to prepare but require occasional regeneration and possibly addition of the coating agent to the electrolyte solution for the separation. Chemically bonded capillaries generally exhibit longer lifetimes and require less maintenance than dynamically coated columns. On the other hand, column preparation is more complex and time consuming, and column reproducibility may be less than desirable. Chemically bonded capillaries may be difficult or impossible to regenerate once degradation is observed.

8.2.7.1 Dynamic Coatings

Dynamic coatings are typically prepared by rinsing the capillary with a solution of the coating reagent. Suitable coating agents can be arbitrarily categorized as alkylamines and diamines [72,134-136], neutral and ionic polymers [132,133,137], cationic surfactants [72,138] and polyelectrolyte multilayers [139-141]. Simple alkylamines and diamines (e.g. triethylamine, triethanolamine, 1,4-diaminobutane, etc.) are only moderately effective at suppressing the electroosmotic flow compared to quaternary amines (e.g. 1,6-bis[trimethylammonium]hexane dibromide), commonly used for the separation of inorganic and small organic anions. The adsorption of charged polymers introduce positive or negative charges on the capillary wall, thus changing the direction and/or the magnitude of the electroosmotic flow, as well as the analyte wall interactions in the separation capillary. Reversed electroosmotic flow can be achieved by coating capillaries with strong cationic polymers (e.g. poly[dimethyldiallylammonium chloride], poly[ethyleneimine], polybrene $\{-[N^+(CH_3)_2(CH_2)_6N^+(CH_3)_2(CH_2)_3-]_n-$, etc). Polyelectrolyte multilayer coatings with a negative charge are formed by initially adsorbing a cationic polymer on the capillary wall (e.g. polybrene) with subsequent dynamic coating with an anionic polymer (e.g. poly[vinylsulfonate]) with a high charge density compared with the cationic polymer. A significant improvement in the long-term stability of the capillaries is obtained by adding a small amount of anionic polymer (e.g. 0.01 % w/v) to the electrolyte solution used for the separation. Importantly, these coatings provide stable and high electroosmotic flow at low pH. Neutral polymer coatings can reduce electroosmotic flow, but more importantly, diminish wall interactions with biopolymers. Biopolymers interact with the column wall by electrostatic, hydrophobic and polar interactions. Hydrophilic layers are generally compatible with biopolymers but usually afford less stable coatings. Typical coating agents include poly(saccharides) and their derivatives, poly(vinyl alcohol), poly(ethylene glycol), poly(ethylene oxide), and poly(dimethylacrylamide). Poly(acrylamide), commonly used to prepare chemically bonded surfaces, is not strongly adsorbed by the silica

surface. Copolymers of acrylamide and hydrophilic monomers with an alkyl backbone bearing glucose, for example, exhibit improved wall adhesion and are suitable for the separation of proteins [137]. Neutral hydrophilic coatings are generally preferred for the separation of proteins but given the wide range of properties exhibited by different biopolymers, there is no single coating that is suitable for all separations. Single chain (e.g. tetradecyltrimethylammonium bromide) and double chain (e.g. didodecyltrimethylammonium bromide) surfactants are widely used for control of electroosmotic flow for anion separations. The negatively charged silica surface results in a surface excess of surfactant leading to micelle formation at the surface, even when the surfactant concentration is below the bulk solution critical micelle concentration. Surface aggregation of surfactant molecules is credited with control of the electroosmotic flow. A mixture of cationic and zwitterionic surfactants allows fine-tuning of the electroosmotic flow. The zwitterionic surfactant has a net zero charge, and in effect, dilutes the positive charge at the capillary surface. Double chain surfactants form more stable coatings than single chain surfactants.

8.2.7.2 Chemically Bonded Layers

The silanol groups on the fused-silica surface provide attachment sites to bond simple ligands or polymeric layers to the column wall [132,133]. Some polymers, such as poly(vinyl alcohol), can be thermally annealed to the silica surface, but a more general approach is provided by silanization. Reaction of the capillary wall with an organotrialkoxysilane, for example, allows layers with an aminopropyl or spacer bonded propanediol group to be prepared [142]. Chemically modified surfaces of this type have a restricted application range, as well as poor stability at high pH, and a limited ability to control the electroosmotic flow. Surface reactions with ω -iodoalkylammonium salts are effective over a wide pH range at reducing electroosmotic flow and decreasing protein adsorption [143]. In this case, the salt is attached to the column wall by alkylation, involving reaction of the iodide group with ionized surface silanol groups. In addition, electrostatic interactions between ionized silanol groups and the quaternary nitrogen atoms, and hydrogen bonding between silanol groups and tertiary nitrogen atoms, contribute to the layer stability.

A common approach for the preparation of chemically bonded layers is based on a series of sequential reactions. To achieve an optimum coating the capillary surface is first cleaned, activated by etching (treatment with 1.0 M NaOH for 30 min) and/or leaching (treatment with 0.1 M HCl at 70°C for 2 h), washed and dehydrated (160°C overnight with a flow of nitrogen) [144,145]. The capillary wall is then reacted with a silanizing reagent (e.g. 7-oct-1-enyltrimethoxysilane, 3-methacryloxypropyltrimethoxysilane, 3-glycidioxypropyltrimethoxysilane, etc.) under defined conditions (solvent, temperature, time, catalyst) optimized to yield a dense surface coverage of siloxane-bonded groups [145-150]. Poly(ether) chains of different length and with different substituent groups can be covalently bonded to a sublayer prepared from 3-glycidioxypropyltrimethoxysilane. Poly(sacharides) such as hydroxypropylcellulose can be covalently bonded to a sublayer prepared from iso-

cyanatopropyltriethoxysilane or 3-glycidoxypropyltrimethoxysilane. Coatings containing poly(acrylamide), poly(2-hydroxyethylmethacrylate), poly(vinylpyrrolidone), etc., can be prepared by *in situ* copolymerization of surface-bonded acryl or vinyl groups and a suitable monomer (e.g. acrylamide) or reacted with a polymer. Wall coatings with a linear poly(acrylamide) layers are the most widely used. The capillary is filled with a solution of acrylamide or dimethylacrylamide, initiator (N,N,N',N'-tetramethylethylenediamine) and catalyst (ammonium persulfate) and polymerized under controlled conditions. Coated columns can also be prepared by statically coating the capillary with a solution containing a hydrophilic polymer [e.g. poly(ethyleneimine), poly(ethylene glycol), hydroxypropylcellulose, etc.), a derivatizing reagent (hexamethyldisilazane) and a crosslinking agent (e.g. dicumyl peroxide) [151].

The problem of protein-wall interactions still limits many capillary electrophoretic applications in routine biochemical analysis. Unstable migration times, poor column-to-column reproducibility, instability of siloxane-bonded layers at high pH and electric field strengths, and limited column lifetimes remain persistent problems, partially ameliorated by recent advances in coating technology, but not completely solved.

8.3 MICELLAR ELECTROKINETIC CHROMATOGRAPHY

Micellar electrokinetic chromatography (MEKC) extends the application range of capillary electrophoresis by providing a mechanism for the separation of neutral as well as ionized solutes [151-160]. The feature that distinguishes micellar electrokinetic chromatography from other capillary-electromigration separation techniques is the addition of a surfactant above its critical micelle concentration to the separation buffer. In an electric field, neutral solutes are separated based on their distribution between the electrolyte solution and the surfactant micelles. The micelles form a pseudostationary phase, which unlike conventional chromatographic systems, is not stationary, but moves with a migration velocity and/or direction that is different to the mobile phase. Consequently, separations are constrained to occur within a migration window defined by the difference in the migration velocity of the electrolyte solution, identical to the electroosmotic flow velocity, and the effective migration velocity of the micelles, Figure 8.4. In other words, all solutes that reside exclusively in the mobile phase (electrolyte solution) are unseparated and elute from the column at time t_{eo} , and those solutes that reside exclusively in the pseudostationary phase are unseparated and elute from the column at time t_{mc} . Solutes that are distributed between the two phases may be separated with a characteristic retention times t_R , in which $t_{eo} < t_R < t_{mc}$. The importance of the migration window, is that it defines the peak capacity for the separation system. Selectivity, on the other hand, depends largely on the difference in distribution constants between the two phases for neutral solutes, and the difference in distribution constants and effective electrophoretic mobility for ionized solutes. Favorable kinetic properties result in high efficiency (> 200,000 theoretical plates) and a reasonable peak capacity, even for systems with a narrow migration window. A successful separation, therefore,

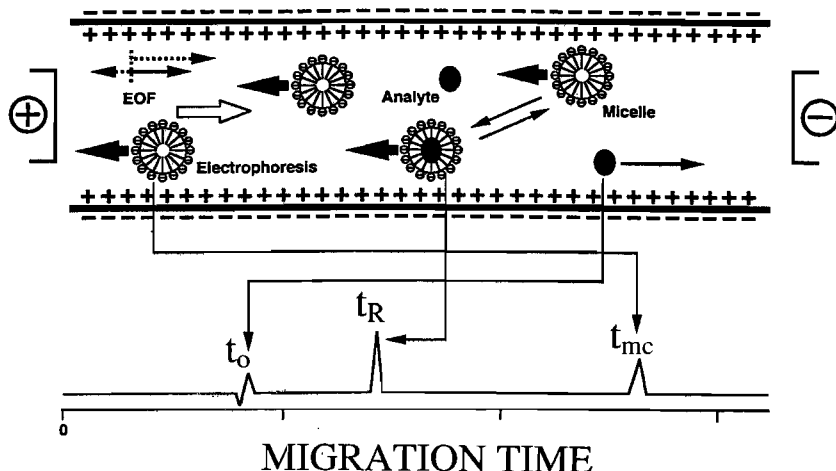


Figure 8.4. Schematic representation of a micellar electrokinetic chromatography system containing an anionic surfactant. (From ref. [160]; ©Elsevier).

mainly depends on the choice of surfactant system, operation under conditions resulting in an acceptable migration window, and maintenance of experimental conditions that provide high kinetic efficiency. In turn, these parameters are influenced by the field strength, buffer composition, ionic strength, and pH; capillary surface characteristics; temperature; and choice of additive (e.g. organic solvent, complexing agent, etc.) and its concentration.

8.3.1 Theoretical Principles

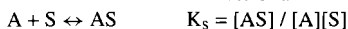
The general theory of micellar electrokinetic chromatography represents a confluence of chromatographic and electrophoretic principles. The expressions for electrophoretic mobility under different separation conditions are summarized in Table 8.4 [161,162]. These relationships allow the determination of the critical micelle concentration and equilibrium distribution constants for solute-micelle association complexes under typical conditions for micellar electrokinetic chromatography [60-64,161-164]. These properties change significantly with the composition of the electrolyte solution, and are generally different to common reference values for pure water.

The apparent electrophoretic mobility of an analyte in micellar electrokinetic chromatography depends on three factors: the electroosmotic mobility for the system; the fraction of analyte in the electrolyte solution and its electrophoretic mobility; and the fraction of analyte in the pseudostationary phase, and the electrophoretic mobility of the micelles (assuming that the mobility of the analyte-micelle complex is the same as the micelle). If we introduce the chromatographic retention factor, defined as the ratio of the number of analyte molecules in the pseudostationary phase to the number in the

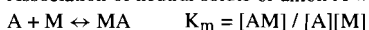
Table 8.4

Electrophoretic mobility of neutral solutes and anions in micellar electrokinetic chromatography

Association of neutral solute or anion A with surfactant monomers S



Association of neutral solute or anion A with surfactant micelles M



The equilibrium between surfactant complexes AS and micelle complexes AM is considered unimportant and the total surfactant concentration $C_T = [S] + [M]$.

(i) $C_T < \text{cmc}$ (critical micelle concentration)

For neutral solutes

$$\mu_{\text{eff}} = K_s[S] / (1 + K_s[S])$$

For anions

$$\mu_{\text{eff}} = \mu_A + K_s[S]\mu_{AS} / (1 + K_s[S])$$

If interactions between A and S are weak corresponding to $K_s \approx 0$

$$\mu_{\text{eff}} \approx 0 \text{ (neutral solutes)}$$

$$\mu_{\text{eff}} \approx \mu_A / (1 + K_s[S]) \text{ or } \mu_{\text{eff}} \approx \mu_A \text{ (if } K_s[S] \ll 1) \text{ (anions)}$$

(ii) $C_T > \text{cmc}$ Above the cmc the surfactant monomer concentration $[S] \approx [\text{CMC}]$

For neutral solutes

$$\mu_{\text{eff}} = (K_s[\text{CMC}]\mu_{AS} + K_m[M]\mu_{MA}) / (1 + K_s[\text{CMC}] + K_m[M])$$

For anions

$$\mu_{\text{eff}} = (\mu_A + K_s[\text{CMC}]\mu_{AS} + K_m[M]\mu_{MA}) / (1 + K_s[\text{CMC}] + K_m[M])$$

If interaction between A and S are weak corresponding to $K_s \approx 0$

$$\mu_{\text{eff}} \approx (K_m[M]\mu_{MA}) / (1 + K_m[M]) \text{ (neutral solutes)}$$

$$\mu_{\text{eff}} \approx (\mu_A + K_m[M]\mu_{MA}) / (1 + K_m[M]) \text{ (anions)}$$

electrolyte solution, then the general equation for the apparent electrophoretic mobility of an analyte can be written as [11,165-168]

$$\mu_{\text{ap}} = \mu_{\text{eo}} + [1 / (1 + k)]\mu_{\text{ep}} + [k / (1 + k)]\mu_{\text{mc}} \quad (8.20)$$

where μ_{eo} , μ_{ep} , and μ_{mc} are the electroosmotic mobility, the electrophoretic mobility of the analyte (which is zero for a neutral compound) and the electrophoretic mobility of the micelles, respectively, and k is the retention factor. Substituting the migration time for the electrophoretic mobility of a neutral compound (Eq. 8.13) in Eq. (8.20), and rearranging, gives

$$k = (t_R - t_{\text{eo}}) / (1 - t_R / t_{\text{mc}})t_{\text{eo}} \quad (8.21)$$

where t_R , t_{mc} and t_{eo} are the analyte migration time, micelle migration time, and the migration time of a neutral solute that does not interact with the pseudostationary phase, respectively. Similarly, the migration time in terms of the retention factor is given by

$$t_R = t_{\text{eo}}[(1 + k) / 1 + (t_{\text{eo}} / t_{\text{mc}})k] \quad (8.22)$$

The only difference between retention in conventional chromatography and micellar electrokinetic chromatography is the extra term in the denominator of Eq (8.22). This is a consequence of the limited migration range in micellar electrokinetic chromatography ($t_{eo} / t_{mc} > 0$). As $t_{mc} \rightarrow \infty$, then Eq (8.22) is equivalent to the general chromatographic retention equation (section 1.4). Suppression or reversal of the electroosmotic flow by using cationic surfactants or coated capillaries, for example, results in a reversal of the normal separation order [166]. The first peak to elute corresponds to t_{mc} and t_{eo} elutes much later, or is not observed at all. For these conditions the migration time for a neutral solute is given by $t_R = t_{mc}(1 + k)$.

The situation is more complex for the separation of ions by micellar electrokinetic chromatography [167-171]. The migration of an ionizable solute is the weighted average of the migration of the charged solute in the buffer, the migration of the solute interacting with the micelles, and the migration of the neutral form of the solute. For anionic micelles the retention factor of the neutral form of a weak acid (HA) is described by

$$k = [1 / (1 - \alpha_A)][(\mu_{ap} - \alpha_A \mu_A) / (\mu_{mc} - \mu_{ap})] \quad (8.23)$$

where α_A is the degree of dissociation (the fraction of all species containing A that exist as A^-), μ_{ap} the apparent electrophoretic mobility of the acid under MEKC conditions, μ_A the electrophoretic mobility of the anion A^- in the electrolyte solution in the absence of surfactant, and μ_{mc} the electrophoretic mobility of the micelles [167]. The migration time

$$t_R = t_{eo}(1 + k) / [1 + \mu_r + (t_{eo} / t_{mc})k] \quad (8.24)$$

and the retention factor

$$k = [t_R(1 + \mu_r) - t_{eo}] / [(1 - t_R / t_{mc})t_{eo}] \quad (8.25)$$

of an ion are given by Eqs. (8.24) and (8.25), respectively, where μ_r is the relative electrophoretic mobility (the ratio of the electrophoretic mobility of the analyte in the electrolyte solution in the absence of micelles and the electroosmotic mobility of the MEKC system) [11,169-171]. Eqs. (8.24) and (8.25) are correct in the absence of specific interactions between ions and the charged micelles, such as ion pair or ion repulsion interactions.

To calculate the retention factor or electrophoretic mobility either marker compounds [172] or a homologous series iterative method is generally used [173,174]. Typical marker compounds for t_{eo} include neutral compounds with strong UV absorption and high solubility in the electrolyte solution (e.g. formamide, thiourea, dimethylsulfoxide). These are added in small amounts to each sample and must not be retained by interactions with the column wall or pseudostationary phase. Alternatively, t_{eo} can be estimated from the first deviation of the baseline of the electropherogram when an

organic solvent of low absorbance (e.g. methanol, acetonitrile, etc.) is injected into the MEKC system. Comparative studies indicate that not all marker compounds elute at the same time, and thus provide a reliable estimate of t_{eo} . The baseline disturbance method and the migration time of formamide were shown to provide a reasonable estimate of t_{eo} for different surfactant types [172]. To determine t_{mc} the marker compound must have acceptable solubility in the electrolyte solution, have useful UV absorption, and a high affinity for the pseudostationary phase. Octylbenzene and dodecanophenone were shown to be suitable compounds for different surfactant types [172]. Alkylbenzenes or alkylaryl ketones are generally used in the iterative method to determine t_{mc} . The homolog with the highest retention is taken as an estimate of t_{mc} and used with Eq. (8.21) to calculate the retention factors for the other homologs. The logarithm of the retention factors is then regressed against the chain length (carbon number) of the alkyl chain and a new estimate of t_{mc} obtained from the best-fit linear model. The new estimate of t_{mc} is used to recalculate the retention factors and the fitting procedure repeated until the difference between consecutive estimates of t_{mc} is acceptable.

The main sources of band broadening in micellar electrokinetic chromatography are longitudinal diffusion and instrumental sources of dispersion, with thermal gradients at high field strengths, and micellar overload at high analyte concentrations as further contributing factors [175,176]. At low field strengths and infinite dilution the contribution from longitudinal diffusion can be calculated from Eq. (8.16) (section 8.2.3) with the effective diffusion coefficient calculated by

$$D = [1 / (1 + k)]D_m + [k / (1 + k)]D_{mc} \quad (8.26)$$

where D_m is the analyte diffusion coefficient in the electrolyte solution (mobile phase) and D_{mc} the micelle diffusion coefficient (assumed to be identical to the analyte-micelle complex). Because D_{mc} is typically an order of magnitude or more less than D_m , analytes spanning the full range of retention factors have values of D that span a similar scale. This has a considerable effect on the separation efficiency. The diffusion coefficients D_m and D_{mc} increase at high field strengths, because of the higher temperature of the electrolyte solution. In addition, electrophoretic mobility increases with temperature owing to a reduction of the solution viscosity. Therefore, both charged and neutral analytes are thermally dispersed by micelles during the time they spend in the pseudostationary phase. In addition, charged analytes are thermally dispersed during the time they spend in the mobile phase. These dispersion sources are relaxed by radial diffusion.

The contribution of longitudinal diffusion to the plate height is the product of the average diffusion coefficient and the migration time. The apparent electrophoretic mobility of an analyte is described by Eq. (8.20). If μ_{eo} and μ_{mc} are affected differently by changing separation conditions, then the migration time of analytes with different retention factors are also affected differently. Such variations cause the migration time to vary with the retention factor independent of the variation of the effective diffusion coefficient with the retention factor. This is an additional source of longitudinal

diffusion with a strong system dependency, which when combined with variation of diffusion coefficients, can explain most of the observations for changes in separation efficiency with experimental variables. Factors which are known to affect the separation efficiency in micellar electrokinetic chromatography include the retention factor, field strength, temperature gradient, concentration of organic solvent, buffer composition and concentration, surfactant concentration, and the polydispersity of the micelles. These factors are often interconnected, and the interpretation of band broadening in terms of system variables is not straightforward.

The general resolution equation for two neutral analytes with similar retention factors in micellar electrokinetic chromatography, is similar to the relationship for chromatography (section 1.6) with an additional term that arises from the limited migration time window [11,12,166,177,178].

$$R_s = (\sqrt{N}/4)(\alpha - 1/\alpha)(k_2/k_1 + 1)(1 - t_{eo}/t_{mc})(1 + k_1 t_{eo}/t_{mc})^{-1} \quad (8.27)$$

where N is the separation efficiency (plate number) and α the separation factor (k_2/k_1). Large values of the retention factor are unfavorable for high resolution, since the last two terms in Eq. (8.27) become almost zero. The optimum value of the retention factor for maximum resolution depends on the ratio of t_{eo}/t_{mc} and is around 0.8-5, or equivalent to $(t_{eo}/t_{mc})^{1/2}$. Outside this optimum range, resolution declines rapidly due mainly to the limited migration window. The size of the migration window can be increased by using mixed micelles or coated capillary columns, decreasing the pH of the electrolyte solution (anionic micelles), or by adding organic solvents or inorganic salts to the electrolyte solution [179-181].

8.3.2 Micelles as Psuedostationary Phases

Surfactants are amphiphilic compounds consisting of a hydrocarbon moiety with a polar or ionic head group. They are conveniently categorized by the charge on the head group as non-ionic, anionic, cationic or zwitterionic surfactants, or by the type of organic moiety as hydrocarbon, bile salts and fluorocarbon surfactants [156,157,182-185]. A general property of all surfactants is the formation of micelles (molecular aggregates) when their solution concentration exceeds a threshold value, called the critical micelle concentration (cmc). The driving force for micelle formation in aqueous solution is the favorable free energy change accompanying segregation of the hydrocarbon-like portion of the surfactant from water by packing hydrocarbon groups into a central core surrounded by the polar head groups. Electrostatic repulsion between head groups in ionic micelles, and the steric repulsion of hydrated head groups in the case of non-ionic micelles, oppose this favorable free energy change. The critical micelle concentration and the size, shape and aggregation number of the micelle, depend on the balance between the factors promoting micelle formation and those opposing it.

The interior portion of a micelle is somewhat fluid and relatively non-polar. It consists of intertwined, randomly orientated, hydrocarbon-like groups, although the intrinsic

viscosity of the micellar core may be an order of magnitude greater than that of a hydrocarbon with a similar chain length. The surface of the micelle consists of the polar head groups, bound counterions, and associated water molecules. The net charge of ionic micelles is less than the aggregation number, indicating that a large fraction of the counterions remain associated with the micelle.

The main factors that affect the critical micelle concentration and the size of ionic micelles, are the type of head group and associated counterions, the size and structure of the hydrocarbon-like moiety, the concentration of added electrolyte, and temperature [182-188]. In aqueous solution, many common ionic surfactants form spherical micelles at concentrations in the range 1 to 10 times the critical micelle concentration. At higher concentrations, or in the presence of electrolytes or organic additives, spherical micelles convert to ellipsoidal (e.g. globular, dumbbell, etc.), rod-like, or other non-spherical forms, in which the surfactant head groups pack closer together than at low concentrations and in the absence of added electrolyte. Normally, spherical and ellipsoidal micelles have low size dispersity, characterized by a narrow range of aggregation numbers. Rod-like micelles can be visualized as having a cylindrical middle part with two spherical endcaps, and are often characterized by a broad distribution of aggregation numbers. Because a micelle has a small volume, it must change into a rod-like micelle to increase its volume-to-surface ratio. Thus, either the addition of electrolyte or increasing the concentration of an ionic surfactant will increase the ionic strength, partially screening repulsive interactions between the ionic head groups, and causing the more densely packed non-spherical micelle forms to become thermodynamically more stable than spherical forms.

Short chain alcohols, and other additives, such as urea, are known to increase the critical micelle concentration of surfactants through their influence on the structure of the solvent [184,185]. The additives are generally not incorporated into the micelles, unlike long chain alcohols, which tend to decrease the critical micelle concentration with increasing concentration through their solubility in the micelle and reduction of electrostatic repulsion between the ionic head groups [189]. At relatively low organic solvent concentration (< 25 % v/v) aggregation of surfactant monomers in aqueous solution is substantially inhibited [190].

The micelle has too small an aggregation number to be considered as a phase in the usual sense, and yet normally contains too many surfactant molecules to be considered as a chemical species. It is this dichotomy that makes an exact theory of solubilization by micelles difficult. The primary theoretical approaches to the problem are based on either a pseudophase model, mass action model, multiple equilibrium model, or the thermodynamics of small systems [191-196]. Technically, bulk thermodynamics should not apply to solute partitioning into small aggregates, since these solvents are interfacial phases with large surface-to-volume ratios. In contrast to a bulk phase, whose properties are invariant with position, the properties of small aggregates are expected to vary with distance from the interface [195]. The lattice model of solute partitioning concludes that virtually all types of solutes should favor the interface over the interior of a spherical micelle. While for cylindrical micelles, the internal distribution of solutes

favors the core, except for non-ideal solutes, which have a greater affinity for the interface. For sodium dodecyl sulfate micelles, low concentrations of both polar and non-polar solutes are solubilized at the interface in preference to the hydrocarbon core of the micelles [196]. A two-state model for micellar solubilization in which it is assumed that a solute may exist in a dissolved state in the micellar core, and in an adsorbed state at the micelle-water interface, was formulated [194]. For the majority of solutes, it was hypothesized that the adsorbed state is primarily responsible for micelle solubilization with higher solute concentrations favoring redistribution to the dissolved state. Another popular theory assumes that the micelle can be divided into two regions; the core region, where only solute molecules are allowed, and the palisade layer, where surfactant molecules are anchored with their head groups at the interface pointing towards water [191]. Depending on the charge density for ionic micelles and the ionic strength of the electrolyte solution, solutes of different polarity are expected to distribute themselves between the micelle core and palisade layer to different extents.

The above theories are useful in shaping expectations for the retention behavior of solutes in micellar electrokinetic chromatography if specific features of the electrophoretic experiment are brought into consideration. Micellar electrokinetic chromatography requires a buffer, the concentration and identity of which affects the shape, size and aggregation number of the micelles. A comprehensive study of the effect of the normal range of experimental variables for sodium cholate micelles indicated quite modest changes in selectivity, suggesting that these considerations do not result in radical changes in separation properties [197]. Typical sample sizes in micellar electrokinetic chromatography are significantly lower than the micelle concentration and solute distribution between the micelles and electrolyte solution occurs within the Henry's law region [185]. At relatively high solute concentrations, penetration of the hydrocarbon core region of the micelle is the only reasonable explanation for observations made in a large number of spectroscopic experiments and practical applications of micelles in detergency and synthetic chemistry. In the absence of this driving force, however, these results may be misleading when imposed on micellar electrokinetic chromatography. In addition, solute-solute interactions within the interior of the micelles that contribute to solubilization at high solute concentrations are unlikely to be relevant at the low analyte concentrations typical of micellar electrokinetic chromatography.

The structural heterogeneity of micelles and their high surface-to-volume ratios are the primary characteristic features that distinguish the sorption behavior of micelles from those of bulk solvents. These properties fuel the speculation that solutes of different polarity are localized in different regions (microenvironments) of the micelles [189,198]. However, studies of a large and varied group of solutes indicated that the sorption properties of micelles could be explained by assuming either an average or single sorption environment [199]. In addition, it was shown that the sorption environment was considerably more polar than could be explained by considering a hydrocarbon solvent as a model for the micelle core. This, and further studies, led to the proposal of an interphase model to explain selectivity differences resulting from

analyte-micelle interactions in micellar electrokinetic chromatography [185,200,201]. The interphase is defined as the region surrounding the core of the micelle containing the polar head groups and possibly immediate-neighbor segments of the hydrocarbon chain, as well as components of the electrolyte solution, organized into a loose structure owing to their proximity to the micelle surface. The actual boundaries between the core of the micelle and the interphase region, and the interphase region and the bulk electrolyte solution, are not well defined, and may change as the electrolyte composition is varied. The composition of the interphase region is not necessarily homogeneous, but since the interphase region is thin, solutes can readily explore all microenvironments, resulting in an averaging effect when macroscopic properties are determined. The electrolyte composition in the interphase region is probably different from the bulk electrolyte solution, and is controlled by short-range electrostatic surface forces. Likewise, the composition of the interphase region may be different to the bulk electrolyte solution when organic additives are present in the electrolyte solution, due to selective solvation of the micelle surface by the additive. The main tenet of the interphase model is that retention in micellar electrokinetic chromatography results from the difference in solvation properties of the interphase region and the bulk electrolyte solution. In the interphase model, it is likely that the micelle core does not play a significant role in retention beyond its role in stabilizing the micelle structure.

8.3.3 Surfactant Micelles

Only a small number of surfactants are used to any significant extent in micellar electrokinetic chromatography [156,157,182]. They can be categorized as anionic surfactants containing sulfate, sulfonate or carboxylate head groups, bile salts, cationic surfactants containing quaternary ammonium head groups, and miscellaneous neutral and zwitterionic surfactants employed as mixed surfactant micelles (section 8.3.3.1). Useful surfactants should have a low critical micelle concentration and a low Kraft point. Typical concentrations of ionic surfactants used in micellar electrokinetic chromatography are 10 - 200 mM. Higher ionic surfactant concentrations are undesirable because they result in excessive current and high solution viscosity. In practice, this means that the critical micelle concentration of ionic micelles should be less than 200 mM, and to provide a dynamic concentration range to optimize retention factors, a critical micelle concentration less than 10 mM is preferable. The Kraft point is the temperature at which the solubility of the surfactant is equal to the critical micelle concentration. Below the Kraft point the surfactant will precipitate from solution. Surfactants with a Kraft point above room temperature are inconvenient for general use. In addition, the surfactant must be available in a pure form, have good solubility in electrolyte solutions, and should be stable over the pH range required to maintain a useful electroosmotic flow velocity (for anionic surfactants this is usually pH 6-9). It should also have low UV absorbance, since absorption detection is the most common detection technique for micellar electrokinetic chromatography. The characteristic properties of some representative surfactants are summarized in Table 8.5. The critical micelle concentration is indicated

Table 8.5
Characteristic properties of some common surfactants

Surfactant	CMC* (mM)	Aggregation number	Kraft point (°C)
<i>(i) Anionic</i>			
Sodium dodecyl sulfate	8.1	62	9
Sodium dodecanesulfonate	7.2	54	37.5
Sodium N-lauroyl-N-methyltaurine	8.7		<0
Lithium perfluorooctanesulfonate	6.7		
<i>(ii) Bile salts</i>			
Sodium cholate	13-15	2-4	
Sodium deoxycholate	4-6	4-10	
Sodium taurocholate	10-15	5	
<i>(iii) Cationic</i>			
Tetradecyltrimethylammonium bromide	3.5	75	
Hexadecyltrimethylammonium bromide	0.92	61	
<i>(iv) Zwitterionic</i>			
N-Dodecyl-N,N-dimethylammino-3-propanesulfonate (Sulfobetain SB-12)	3.3	55	<0
3-[(3-cholamidopropyl)dimethylammonio]-3-propanesulfonate (CHAPS)	8	10	
<i>(v) Non-ionic</i>			
Polyoxyethylene [23] dodecanol (Brij 35)	0.09		40
Polyoxyethylene [20]-sorbitanmonolaurate (Tween 20)	0.95		

*Critical micelle concentration in water

for water as solvent, and is only a rough guide to the real value for electrolyte solutions (section 8.3.1).

The solvation parameter model (section 1.4.3) provides a convenient framework for discussion of the sorption properties of surfactant micelles under typical conditions for micellar electrokinetic chromatography, Table 8.6 [201]. Suitable experimental conditions for determining system constants have been recommended [200,202,203]. For comparison the surfactants are grouped into alkyl sulfates and sulfonates [197,200,204-209], bile acids [197,200,210], miscellaneous anionic surfactants [205,206,211], and cationic surfactants [197,200,202] in Table 8.6. In general terms, the ease of cavity formation and dispersion interactions (m system constant) and electron lone pair interactions (r system constant) favor solubilization by the pseudostationary phase. Solute interactions of a dipole-type and hydrogen bonding are more favorable in the electrolyte solution, and oppose transfer to the pseudostationary phase. General exceptions are the cationic surfactants, which form micelles that are stronger hydrogen-bond bases than the electrolyte solution (a system constant is positive) and the perfluorooctanesulfonate surfactant, whose solubilizing properties are different to the other surfactants because of the inductive effect of the electronegative fluorine atoms. Two sets of values are given for the system constants for lithium perfluorooctanesulfonate in Table 8.6 because the literature values are in conflict, but demonstrate similar trends [197,208]. The general trends in system constants for reversed-phase liquid chromatography (section 4.3.1.3)

Table 8.6

System constant ratios from the solvation parameter model for surfactant micelles
(Temperature = 20-25°C except were noted)

Surfactant	Abbreviation	System constant ratios				
		<i>m</i>	<i>r/m</i>	<i>s/m</i>	<i>a/m</i>	<i>b/m</i>
<i>(i) Alkane sulfates and sulfonates</i>						
Sodium octyl sulfate	SOS	2.85	0.16	-0.11	-0.04	-0.66
Sodium decyl sulfate	SDecS	2.69	0.12	-0.09	0	-0.59
Sodium dodecyl sulfate (36°C)	SDS	2.98	0.12	-0.14	-0.08	-0.64
		2.86	0.09	-0.11	-0.05	-0.59
Lithium dodecyl sulfate (35°C)	LDS	2.81	0.13	-0.15	-0.07	-0.55
		3.01	0.10	-0.12	-0.07	-0.59
Magnesium dodecyl sulfate (35°C)	Mg(DS) ₂	3.02	0.09	-0.14	-0.09	-0.62
Copper dodecyl sulfate (35°C)	Cu(DS) ₂	3.05	0.11	-0.17	-0.09	-0.63
Sodium dodecyl sulfonate (36°C)	SDSu	2.84	0.12	-0.15	-0.01	-0.63
Sodium tetradecyl sulfate (35°C)	STS	3.01	0.09	-0.11	-0.06	-0.60
Lithium perfluorooctanesulfonate	LPFOSu	2.20	-0.11	0	-0.42	0
		2.36	-0.29	0.20	-0.34	-0.25
Sodium N-lauroyl-N-methyltaurine	SLMT	2.88	0.18	-0.12	0.14	-0.82
Sodium N-dodecanoyl-N-methyltaurine	SDMT	3.07	0.23	-0.16	0.07	-0.84
<i>(ii) Bile acids</i>						
Sodium cholate	SC	2.45	0.26	-0.19	0	-0.93
Sodium deoxycholate	SDC	2.67	0.25	-0.18	0	-0.93
Sodium taurocholate	STC	2.43	0.25	-0.14	0	-0.85
Sodium taurodeoxycholate	STDC	2.62	0.26	-0.17	0	-0.83
<i>(iii) Miscellaneous anionic surfactants</i>						
Sodium N-lauroylsarcosinate	SLN	2.98	0.14	-0.12	0.15	-0.78
Sodium N-myristoylsarcosinate	SMN	2.99	0.16	-0.14	0.15	-0.82
Sodium N-parmitoylsarcosinate	SPN	3.11	0.14	-0.14	0.15	-0.83
Sodium N-lauroyl-N-methyl-β-alaninate	ALE	2.92	0.15	-0.13	0.17	-0.83
Sodium dodecoxycarbonylvaline	SDCV	2.99	0.14	-0.19	0.05	-0.81
Sodium dodecylcarboxylate (36°)	SDCA	2.96	0.05	-0.13	0.08	-0.60
Sodium dodecylphosphate (36°)	SDP	3.01	0.08	-0.18	0.05	-0.66
Sodium lauryl sulfoacetate (36°)	SLSA	2.97	0.16	-0.13	0.04	-0.82
<i>(iv) Cationic surfactants</i>						
Tetradecyltrimethylammonium bromide	TTAB	2.99	0.10	-0.07	0.29	-0.91
Hexadecyltrimethylammonium bromide	CTMAB	3.40	0.18	-0.16	0.17	-0.91

and micellar electrokinetic chromatography are similar, but their ranges do not completely overlap, providing for complementary separation properties [201,212]. One feature that stands out in Table 8.6 is the narrow range of *m* system constants for all surfactant types. This is probably a reflection of the need for a minimum cohesive energy difference for aggregation of surfactant monomers.

Selectivity differences for the alkane sulfates and sulfonates are not particularly sensitive to changes in the identity of the counterion or the alkyl chain length [204,209]. Sodium dodecyl sulfate is representative of this group. On the other hand, the perfluorooctanesulfonate and N-alkyl-N-methyltaurine surfactants afford different selectivity to sodium dodecyl sulfate [197,202,208]. Lithium perfluorooctanesulfonate has differ-

ent selectivity for electron lone pair interactions (the only negative r system constant), is the most dipolar (the only positive s system constant) and is the strongest hydrogen-bond acid and weakest hydrogen-bond base of all surfactants in Table 8.6. The N-alkyl-N-methyltaurine surfactants are stronger hydrogen-bond bases and weaker hydrogen-bond acids than sodium dodecyl sulfate. Their properties are similar to the cationic surfactants, although tetradecylammonium bromide is a stronger hydrogen-bond base than the N-alkyl-N-methyltaurine surfactants. The bile acids are similar as a group with different selectivity to sodium dodecyl sulfate. They are more cohesive, stronger hydrogen-bond bases and weaker hydrogen-bond acids [197,201]. The N-alkylsarcosinates have similar sorption properties to the N-alkyl-N-methyltaurine surfactants. The dodecylcarboxylate and dodecylphosphate surfactants are slightly more hydrogen-bond basic than sodium dodecyl sulfate, and could be useful for optimizing the peak position of solutes with different hydrogen-bond acidity.

8.3.3.1 Mixed Surfactant Micelles

The retention properties of single surfactant micelles can be modified by forming mixed micelles using surfactants with different solvation properties or by addition of organic additives solubilized by the micelles. Surfactants with a zero net charge, such as non-ionic and zwitterionic surfactants, are unsuitable for the separation of neutral compounds because their mobility is identical to the electroosmotic mobility. When added to an ionic surfactant, mixed surfactant micelles are formed with an overall net charge and adjustable selectivity [213-216]. Non-ionic and zwitterionic surfactants as a component of mixed surfactant micelles allow changes in selectivity, phase ratio, and separation time without affecting the operating current, and usually without degrading the separation efficiency in micellar electrokinetic chromatography. Mixed surfactant micelles also provide a mechanism for expansion of the migration time window to increase the peak capacity of the separation system [180,214]. The influence of the mole fraction ratio of the non-ionic surfactant Brij 35 on the selectivity of mixed surfactant micelles containing sodium dodecyl sulfate [207,217], or sodium N-dodeconyl-N-methyltaurine [218], produced similar trends with only a modest change in retention (e.g. Figure 8.5). The addition of Brij 35 results in small changes in cohesion and the importance of dipole-type and electron lone pair interactions for the mixed micelles. The variation of hydrogen bonding interactions is more significant, and explains the main change in selectivity with micelle composition. Micelles formed from surfactant mixtures with the same formal charge are also suitable for selectivity optimization [208,210]. The main limitation of mixed surfactant micelles for selectivity optimization is the small number of surfactants with significantly different solvation properties, as well as the formation of separate rather than mixed micelles at high mixing ratios [208,219].

Typical water miscible organic solvents (e.g. methanol, acetonitrile, etc.) affect selectivity in micellar electrokinetic chromatography in a manner similar to reversed-phase liquid chromatography. The range of composition variation, however, is restricted by instability of the micelles at moderate concentrations of organic solvents, and

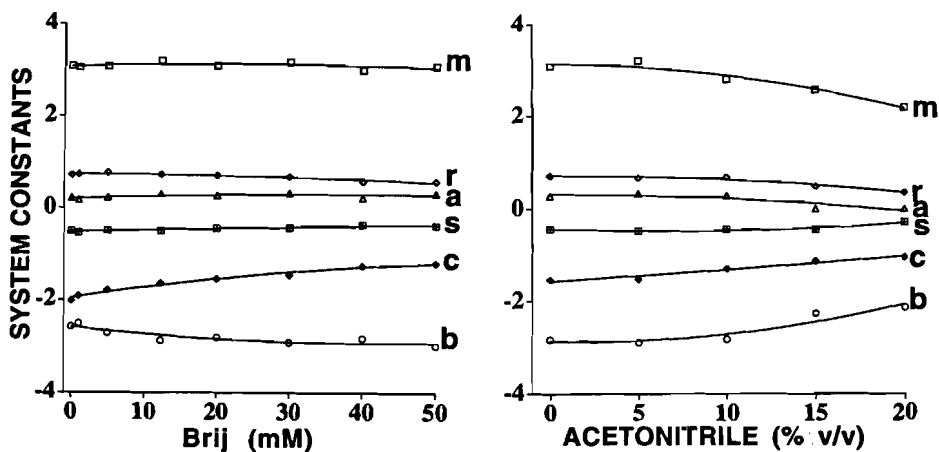


Figure 8.5. Plot of the system constants (solvation parameter model) against composition for a mixed micelle electrolyte solution containing 50 mM sodium N-dodecyl-N-methyltaurine and different amounts of the non-ionic surfactant Brij 35 (polyoxyethylene [23] dodecyl ether) (Left). Plot of the system constants for a mixed micelle buffer containing 50 mM sodium N-dodecanoyl-N-methyltaurine and 20 mM Brij 35 against the volume fraction of acetonitrile added to the electrolyte solution (Right). System constants: m = difference in cavity formation and dispersion interactions; r = difference in electron lone pair interactions; s = difference in dipole-type interactions; a = difference in hydrogen-bond basicity; and b = difference in hydrogen-bond acidity. (From ref. [218]; ©Royal Society of Chemistry).

separation times are compromised by reduction of the electroosmotic flow [48,218,220]. Changes in selectivity are consistent with the view that organic solvents moderate the characteristic solvophobic properties of the aqueous electrolyte while having minimal effect on the pseudostationary phase. Higher molecular mass alcohols, alkyl polyols and neutral polar compounds are solubilized by the micelles forming mixed micelles with similar general properties and limitations to the mixed surfactant micelles [189,218,221]. Urea provides further possibilities for adjusting selectivity by increasing the solubility of water insoluble compounds [177,222].

8.3.4 Polymeric Micelles

Typical polymeric pseudostationary phases include micelle polymers, polymeric surfactants, water-soluble anionic siloxanes and dendrimers [223-231]. Micelle polymers [e.g. poly(sodium 10-undecylenate), poly(sodium 10-undecenylsulfate), poly(sodium undeconylvalinate), etc.] are synthesized from polymerizable surfactant monomers at a concentration above their critical micelle concentration. These polymers have similar structures to micelles without the dynamic nature of the micelle structure. Polymeric surfactants are polymers with surfactant properties [e.g. acrylate copolymers, such as 2-acrylamide-2-methyl-1-propanesulfonic acid and alkyl methacrylamide, alkyl methacrylate or alkyl acrylate, poly(allylamine)-supported phases, poly(ethyleneimine), etc.]. Water-soluble anionic siloxane polymers are copolymers of alkylmethylsiloxane

and methylsiloxane monomers containing ionic groups [227]. Dendrimers are highly branched polymers synthesized from a core structure in a multi-step sequence. The resulting macromolecules have a specific mass and a uniform size with numerous chain-ends that can be functionalized to introduce ionic groups.

Polymeric micelles form stable pseudostationary phases with a critical micelle concentration of virtually zero (aggregation number of 1), and are tolerant of high organic solvent concentrations in the electrolyte solution. Mass transfer kinetics are slow compared with conventional surfactant micelles, and peak distortion from mass overloading is a problem for some polymer compositions. Preliminary studies indicate that polymeric surfactants are effective pseudostationary phases in micellar electrokinetic chromatography, but only a limited number of practical applications have been demonstrated, and uptake has been slow.

8.3.5 Microemulsions

Microemulsions are stable solutions containing dispersed nanometer-sized droplets of an immiscible liquid [232-234]. Microemulsions used in micellar electrokinetic chromatography (commonly called microemulsion electrokinetic chromatography, MEEKC), are a continuous dispersion of charged oil droplets in an aqueous electrolyte. A microemulsion is formed from an organic solvent of low water solubility (e.g. octane, heptane, diethyl ether, octanol, etc.), a charged surfactant (e.g. sodium dodecyl sulfate) and a cosurfactant (e.g. butanol, 2-ethoxyethanol, tetrahydrofuran) in an aqueous buffer. The organic solvent and cosurfactant form the core of the droplet solvating a part of the hydrophobic portion of the surfactant with its charged head group located in the electrolyte solution. At least some of the cosurfactant molecules are arranged with their polar groups located at the organic solvent-electrolyte solution interface. Stable microemulsions are formed over a narrow composition range for the main components, although a variety of cosurfactants and anionic and cationic surfactants alone or mixed with zwitterionic and non-ionic surfactants can be used [235,236]. Practical applications of microemulsion electrokinetic chromatography are dominated by the microemulsion formed by 0.8% (w/w) n-octane, 6.6% (w/w) n-butanol, 3.3% (w/w) sodium dodecyl sulfate and 89.3% (w/w) of 10-20 mM pH 8.5-9.5 borate or phosphate buffer [237,238]. Typical operating conditions are similar to those used for micellar electrokinetic chromatography with a strong electroosmotic flow towards the cathode and a slower electrophoretic mobility of the charged oil droplets towards the anode. Neutral compounds are separated by differences in their distribution constants between the oil droplets and the electrolyte solution. Charged analytes are separated by their electrophoretic mobility in the electrolyte solution and ion pair and ion repulsion interactions with the charged oil droplets.

The oil droplets are coated with a surfactant to reduce the surface tension between the two immiscible liquids allowing emulsion formation. The charge repulsion between the head groups of ionic surfactants prevents efficient packing of surfactant around the oil drop and opposes emulsion formation by restricting reduction of the surface

tension. A cosurfactant, such as a medium-chain-length alcohol, is therefore essential for the formation and increased stability of the microemulsion. The cosurfactant bridges the oil and water interface and further reduces the surface tension of the system to near zero. High surfactant concentrations increase the migration time of neutral solutes owing to the increase in charge density on the oil droplets. Increasing the surfactant chain length stabilizes the microemulsion by reducing the droplet size dispersion. Increasing the concentration of cosurfactant results in an increase in the droplet size, a lower charge density on the oil droplet, and usually a reduction in the migration time for neutral compounds. The concentration range over which stable and useful microemulsions are formed is narrow, and consequently the range of solvation properties presented by microemulsions is limited. Other properties desirable in a microemulsions for microemulsion electrokinetic chromatography are long term stability, low polydispersity, and transparency to UV-visible light.

Although the migration time is affected by the concentration and type of organic solvent chosen to form the oil drop, it plays only a minor role in controlling selectivity compared with the concentration and identity of the surfactant and cosurfactant. The addition of organic solvents to the buffer changes the migration time because of its effect on the electroosmotic flow velocity, and can alter selectivity by increasing the solubility of compounds of low water solubility. Selectivity changes are limited by demixing of microemulsions for solvents such as methanol (< 8% v/v) or acetonitrile (<12 % v/v) for the sodium dodecyl sulfate/n-butanol/n-octane microemulsion. Larger amounts of 2-propanol can be tolerated because it functions as a cosurfactant [235]. Varying the pH of the buffer affects the migration time because of its effect on the electroosmotic flow velocity, and the selectivity of weak acids and bases through changes in the degree of dissociation.

Microemulsion and micellar electrokinetic chromatography are suitable for similar separations, although microemulsion electrokinetic chromatography has been recommended for the separation of analytes of low water solubility [239,240]. Microemulsion electrokinetic chromatography provides more robust separation conditions and improved compatibility with complicated matrices by minimizing matrix precipitation. Microemulsion electrokinetic chromatography also affords a useful surrogate chromatographic model for estimating octanol-water distribution constants, an indication of the similarity of the partitioning properties of the sodium dodecyl sulfate/n-butanol/n-octane microemulsion and wet octanol [241,242]. The separation efficiency of microemulsion electrokinetic chromatography is fully equivalent to micellar electrokinetic chromatography and sometimes better.

8.3.6 Inclusion Complexes

Macrocyclic compounds have been used as additives in micellar electrokinetic chromatography where their ability to modify selectivity by formation of inclusion complexes with a variety of compounds and ions of low water solubility is their most important property [55,243-245]. Neutral cyclodextrins in a buffered micelle solution are

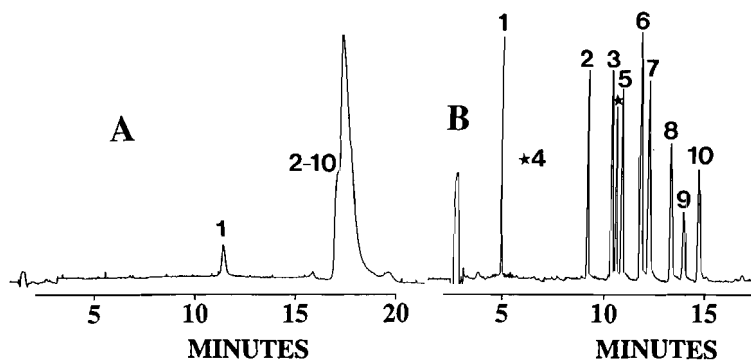


Figure 8.6. Separation of equine estrogens by micellar electrokinetic chromatography using (A) 50 mM sodium dodecyl sulfate in a 20 mM pH 8 buffer and (B) the same conditions as (A) with the addition of 20 mM γ -cyclodextrin. (From ref. [185]; ©Elsevier).

suitable for the separation of neutral hydrophobic compounds and peptides with a similar net charge. Hydrophobic compounds distribute themselves between the cyclodextrin and micelles. When the analyte is included in the cyclodextrin cavity, it migrates with the electroosmotic mobility, and when it is incorporated into the micelles, it migrates with the micelle electrophoretic mobility. Accordingly, the differential distribution of the sample between the cyclodextrin and the micelles results in a separation with different selectivity to the micelle system alone. This approach is particularly useful for the separation of structurally similar compounds (e.g. Figure 8.6) and positional isomers [246]. A number of methylated, hydroxypropylated and acetylated cyclodextrin derivatives are available to extend the application range of natural cyclodextrins.

Cyclodextrin derivatives with ionic groups (methyamino, carboxymethyl, sulfate, sulfobutyl, phosphate) [55,247-250], charged calixarenes [251] and charged resorcinarenes [252] have been used as a replacement pseudostationary phases for surfactant micelles. The mode of separation is similar to conventional micellar electrokinetic chromatography but the pseudostationary phase, in this case, is a flux of individual charged particles. Selectivity differences depend on the equilibrium constants for inclusion-complex formation. While not as common, mixtures of neutral and charged cyclodextrins have been used for fine-tuning selectivity. Distribution now takes place between two fluxes of complex-forming particles with different characteristic velocities and possibly different complexing ability. Single-isomer, ionic cyclodextrins have been developed to minimize variation in migration and separation efficiency caused by charge number and charge location dispersion [253-255].

8.4 CAPILLARY ELECTROCHROMATOGRAPHY

Capillary electrochromatography (CEC) combines some aspects of mobile phase control and separation by capillary electrophoresis with the wider range of separation mech-

anisms possible for liquid chromatography [256-265]. Instrument requirements are similar to capillary electrophoresis and columns similar to microcolumn liquid chromatography. The mobile phase flow through the column is maintained by an electric field in contrast to pressure-driven flow in liquid chromatography. This results in a number of advantages, the most important being that the mobile phase velocity is independent of the particle size, and the radial flow profile is essentially flat. This allows longer columns packed with smaller particles to be used, resulting in a higher separation efficiency and peak capacity. In addition, the flat flow profile reduces flow anisotropy and stationary phase mass transfer resistance providing a further increase in the separation efficiency. The separation mechanism for neutral compounds is expected to resemble reversed-phase liquid chromatography. Ionic and ionizable compounds are separated by a combination of electrophoresis and reversed-phase chromatography. The presence of a high-surface area stationary phase allows larger sample amounts to be separated without loss of efficiency compared with capillary electrophoresis. At this time, capillary electrochromatography is largely a research tool, and not all claimed advantages are fully realized. The technique continues to evolve, and should find its way into the main stream of analytical practice when technical issues are adequately addressed.

8.4.1 Theoretical Principles

Columns for capillary electrochromatography consist of a packed bed from the injection end to the bed-retaining frit in front of the detection zone, and an open section from the bed-retaining frit to the outlet buffer reservoir, Figure 8.7 [266-270]. The electroosmotic flow through the packed bed depends on properties of the frit and open section. The mobile phase in the packed bed occupies only a fraction θ_m of the capillary volume. Hence, if the flow is conserved, the mobile phase velocity in the open section is equal to $v_{eo}\theta_m$, and the corrected velocity through the interparticle space in the packed bed, v_{eo}^* , is given by

$$v_{eo}^* = (L_B + [L_O / \theta_m]) / t_{eo} \quad (8.28)$$

where L_B is the length of the packed bed and L_O the length of the open section from the packed bed to the detection zone. For a typical column $L_B \gg L_O$ and $v_{eo} \approx v_{eo}^*$. This correction becomes important when short packed beds are used for fast separations and for detection configurations where the detection zone is removed from the region next to the bed-retaining frit (e.g. some mass spectrometer interfaces). A further problem arises with the determination of the appropriate field strength for the packed section of the column, which will be different from the value calculated from the total column length [267,268]. In this case, the open section of the column is usually of significant length, and the real field strength in the two sections of the duplex are different [266,268].

An apparent retention factor in capillary electrochromatography can be defined in the usual way as $k_{CEC} = (t_R - t_{eo}) / t_{eo}$. In this case, the retention factor only retains the same physical interpretation as the chromatographic retention factor (section 1.4) for neutral

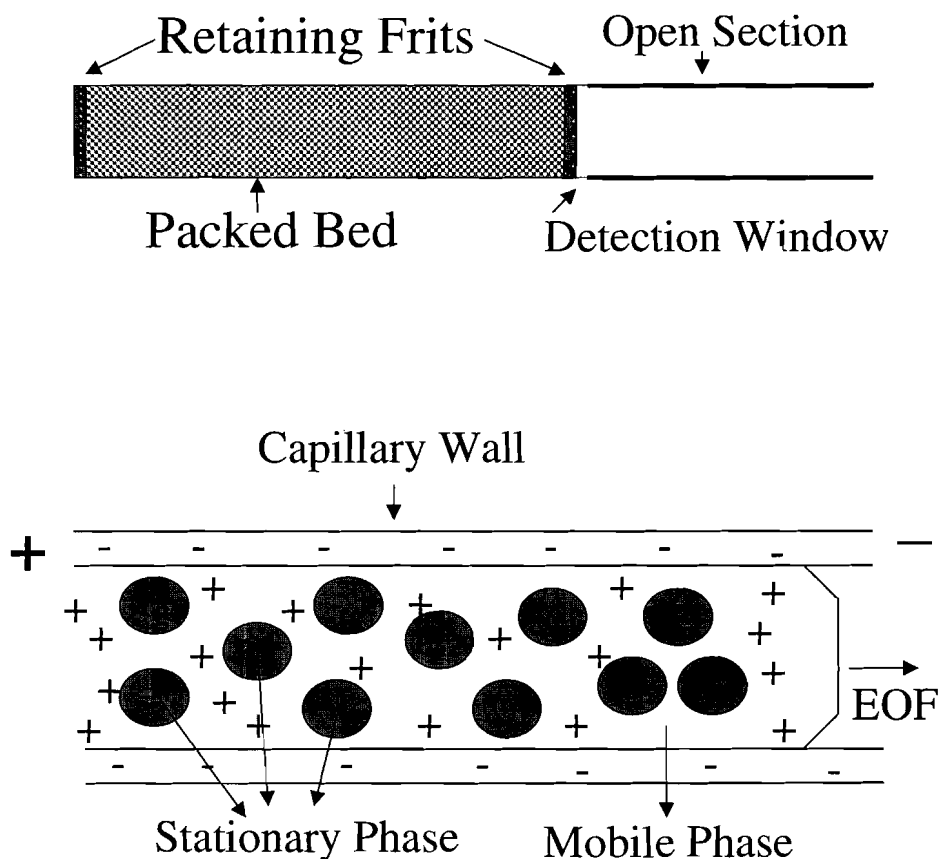


Figure 8.7. Schematic representation of a capillary electrochromatography column consisting of a packed bed and open tubular segment separated by a bed retaining frit (top) and the generation of electroosmotic flow in a packed bed (bottom).

analytes. For ions and partially ionized compounds there is no direct connection with the equilibrium constant because in the mobile phase ions are subjected to additional electrophoretic migration [271]. One manifestation of this difference is that k_{CEC} can have negative values when $t_{\text{eo}} > t_{\text{R}}$. For reversed-phase separations the retention factor in capillary electrochromatography can be related to the liquid chromatographic retention factor, k , by [272-275]

$$k_{\text{CEC}} = k(1 + \mu_r) + \mu_r \quad (8.29)$$

where μ_r is the relative electrophoretic mobility, a signed quantity expressed as the ion electrophoretic mobility divided by the electroosmotic mobility. For neutral solutes, $\mu_r = 0$, and k_{CEC} is identical to the chromatographic retention factor. For ions that

do not interact with the stationary phase, $k = 0$, and $k_{\text{CEC}} = \mu_r$ as expected for separations by capillary electrophoresis. For partially ionized analytes with retention by the stationary phase ($k \neq 0$, $\mu_r \neq 0$), the situation is more complex, as indicated by the product term in Eq. (8.29). For ion exchange stationary phases the situation is further complicated by non-linear effects [270,275-278]. The simulation of k_{CEC} based on Eq. (8.29) requires separate determination of the chromatographic retention factor by liquid chromatography and the electrophoretic migration of the analyte by capillary electrophoresis [273].

Electroosmotic flow in a packed bed of porous particles occurs within the interparticle space and through wide pores by the same mechanism described for open tubular columns (section 8.2.2) [260,264,266,279,280]. The main difference is that the surface of the column packing is mainly responsible for the driving force in capillary electrochromatography. This is so, if only because the surface area of the packing is much larger than the surface area of the column wall. Of fundamental interest is that the general equation for electroosmotic flow, Eq. 8.14, contains no dependence on the particle size as long as significant double layer overlap does not occur within the interparticle space; no dependence on the column length; and the flow profile is flat (Figure 8.7).

In a packed bed of spherical particles the average interparticle channel diameter is about 0.2-0.25 the average particle diameter (d_p). To avoid reduction of the electroosmotic flow the particle diameter should be $\geq 40\delta$, where δ is the double layer thickness. The latter depends on the dielectric constant and ionic strength of the mobile phase, but for typical electrolyte solutions is probably < 10 nm, indicating that $d_p \geq 0.4 \mu\text{m}$ as an acceptable minimum particle size [281,282]. Properties of the stationary phase that promote electroosmotic flow are described by $v_{\text{eo}} = \delta\sigma E / \eta$ where σ is the charge density and η the mobile phase viscosity [262,279,283]. Favorable stationary phase properties depend upon the number of ionic silanol groups or other ionized groups on the stationary phase, the surface area of the particles accessible to the streaming mobile phase, and the type of surface-bound ligands. Silanol groups are weakly ionized at mobile phase pH $< 5-6$. To allow access to a wider pH operating range new stationary phases containing immobilized sulfonic acid or quaternary ammonium groups have been developed [270,283-287].

The combined effect of reducing the particle size, increasing the column length, and the plug-like flow profile is to increase the separation efficiency to values between 200,000 to 500,000 plates per meter, Figure 8.8 [264,266,281,283,288-290]. These values are obtained for columns operated at, or slightly above, the optimum mobile phase velocity (1 – 5 mm/s). In practice, the maximum column length and diameter are dictated by Joule heating, and should be independent of column permeability. Low electrolyte concentrations (1 – 50 mM) and/or zwitterionic buffers are commonly used to minimize temperature gradients. With a maximum potential of 30 kV the column length should be less than about 50 – 60 cm for operation at the optimum mobile phase velocity. The radial temperature profile within the packed capillary leads to a parabolic flow velocity distribution superimposed on the electroosmotic flow plug-like profile

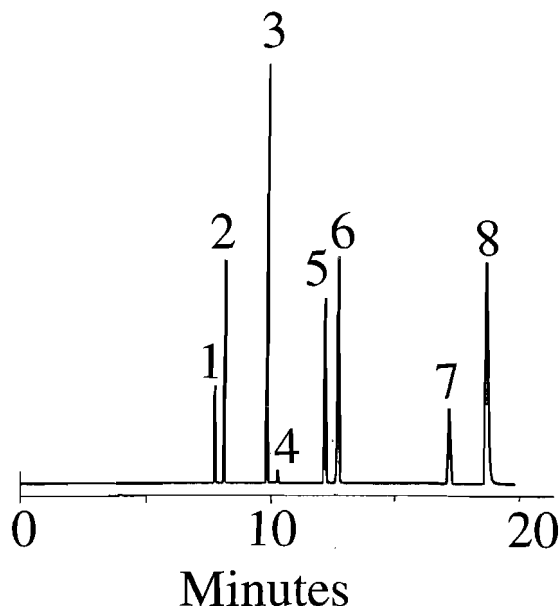


Figure 8.8. Separation of a test mixture on a 50 cm x 50 μm column packed with 1.5 μm Spherisorb ODS-1 with a mobile phase of 70 % (v/v) acetonitrile in a 10 mM pH 9 TRIS buffer at 25°C and voltage 30 kV. Compounds: 1 = acetone; 2 = benzyl alcohol; 3 = dimethyl phthalate; 4 = acetophenone; 5 = anisole; 6 = methyl benzoate; 7 = toluene; and 8 = phenyl benzoate.

leading to a reduction in separation efficiency. With typical electrolyte solutions and column diameters < 150 μm , radial temperature gradients are generally an insignificant source of band broadening. Wider diameter columns are desirable to improve sample detectability and to increase mass loadability for semipreparative applications [291-293]. Thermostatted columns with internal diameters up to 180 μm have been used without loss of separation efficiency [292] and columns of 550- μm internal diameter with useful, if reduced separation efficiency, for semipreparative applications [293]. For example, a 16-cm column of 550- μm internal diameter with 60,000 plates per meter allowed 10-20 μg samples to be separated.

The relationship between separation efficiency and column properties is usually described by the van Deemter equation (section 1.5.2). The low plate height for capillary electrochromatography result from the small contributions from flow anisotropy and resistance to mass transfer. The A (flow anisotropy) and C (resistance to mass transfer) coefficients of the van Deemter equation are smaller by a factor of 2-4 compared with typical values for high-pressure liquid chromatography. The minimum plate height for capillary electrochromatography is typically about 1-2 d_p compared with $2 < d_p < 5$ for high-pressure liquid chromatography. For particles < 1 μm diameter, the contribution of the A and C terms to the minimum plate height are negligible and

the separation efficiency approaches the diffusion limit [281]. Phenomenal separation efficiency, several million plates per meter, was observed for the separation of basic compounds under conditions that resulted in normal values for neutral compounds on ion exchange stationary phases [277,294,295]. The fundamental basis of the focussing mechanism responsible for peak compression is not understood, and the effect is not easily reproduced on different stationary phases, which has delayed exploitation. In addition to the sources of band broadening discussed above, for ions subject to electrophoresis additional band broadening and peak distortion phenomenon, such as electromigration dispersion (sections 8.2.3 and 8.2.5), have to be considered.

The above picture is basically correct but the range of reported plate height values for similar column types is wider than expected [262,266]. Difficulties in sizing small-diameter particles, packing these particles into long capillary columns with a constant permeability, inadequate control of the electroosmotic flow, and extracolumn band broadening are all possible factors contributing to the observed disparity in column properties. Improved instrumentation and column technology should be able to handle these problems.

For porous particles with mean pore diameters less than 10 nm there is virtually no electroosmotic flow through the pores because of electrical double layer overlap. A substantial pore flow is observed for wide pores leading to a significant increase in the separation efficiency [289,296-300]. The separation efficiency is maximized when the velocity of the mobile phase through the pores and the interparticle velocity are nearly identical. In this case, resistance to mass transfer in the stationary phase is virtually eliminated and a strong reduction in flow anisotropy is obtained. For 7- μm particles with pore sizes of 50-400 nm a minimum plate height of 2.3 μm (0.34 d_p) was observed [296]. Wide-pore particles provide an attractive alternative to small-diameter narrow pore and nonporous particles for generating high separation efficiencies and are easier to pack in long capillary columns.

8.4.2 Column Technology

Typical particle-packed columns for capillary electrochromatography are based on silica-based chemically bonded sorbents developed for liquid chromatography (section 4.2) and packing techniques developed for microcolumn liquid chromatography (section 4.5.2) [301-305]. General separations employ fused-silica capillary columns 25-50 cm long and 50-100 μm internal diameter packed with 3-5 μm porous particles with an average pore size of 6-10 nm. Separations are generally performed under reversed-phase conditions with a mobile phase consisting of a buffer (1-100 mM, pH 7-10) with various volumes of acetonitrile or methanol to adjust retention. As well as selectivity, the stationary phase has a major role in establishing and maintaining the electroosmotic flow through the column. A few mixed-mode stationary phases have been developed to separately optimize both properties [283-287]. Other developments include fritless columns based on monolithic stationary phases [306-308] and open tubular columns [309] to tackle the problems associated with sintered-particle frits (section 8.4.2.1). A limited range of packed capillary columns are commercially available,

Table 8.7

Guide for slurry packing fused-silica capillary columns for capillary electrochromatography

(i) Forming the bed-retaining frit

The end of the capillary column is tamped into a paste containing 3- μm silica and 25 % sodium silicate solution (50 mg of silica to 60 μl of silicate solution). A plug of about 0.5 mm is formed by sintering the mixture with a ring heater (section 8.4.2.1). The newly formed frit is tested for pressure stability and permeability before proceeding to the packing step.

(ii) Packing the column

Capillary and slurry reservoir are placed in an ultrasonic bath and packed under conditions similar to those described below:

Reservoir: 5 cm x 2 mm I.D. tube (0.125 in O.D.)

Slurry: \approx 0.1 g/ml of reversed-phase packing material in 2-propanol

Packing solvent: acetone

Time: \approx 30-45 min/m at 600 atm using a gas amplification piston pump

Column: fused silica 50-150 μm I. D. at least 10 cm longer than the required column length

Once the column is packed the pump is switched off and the column allowed to slowly return to atmospheric pressure. An optical microscope is used to check the homogeneity of the bed structure.

(iii) Forming the outlet frit

The capillary is flushed with water by connection to a high-pressure micropump to displace the packing solvent from the column bed. The outlet frit is formed by the sinter method at least 10 cm from the end of the packing while water is flowing through the column. The bed-retaining frit is cut off and excess packing material displaced from the other end of the column by pumping water through the column at an intermediate pressure from the other end.

(iv) Forming the inlet frit

The inlet frit is formed at the desired bed length by the sinter method with water flowing through the column. Excess packing material is removed from the outlet end by pumping mobile phase through the column from the outlet end at an intermediate pressure.

(v) Forming the detector window

A detection window 2-5 mm long is formed 1-2 mm away from the outlet frit by removing the protective polyimide capillary coating with a ring burner.

(vi) Conditioning the column

Flush for several hours at normal flow rates with the mobile phase to be used for the separation. Further condition on the capillary electrochromatography instrument with a stepwise increase in the operating voltage until a stable and adequate electroosmotic flow is obtained.

but most studies until now used self-prepared columns with the aim of advancing column technology, perceived as one of the weak links restraining the general development of capillary electrochromatography [260-264].

8.4.2.1 Packed Capillary Columns

Columns are packed in a multistep process using liquid or supercritical fluid slurry packing methods that differ little in outline from techniques used to prepare packed capillary columns for liquid chromatography (section 4.5.2) [301,302,305,310-315]. A general outline for packing capillary columns with particles $\geq 3 \mu\text{m}$ in diameter is presented in Table 8.7. Since there is no single optimal column packing method, and individual sorbents require modification to generic methods on a case by case basis, Table 8.7 is only intended as a general guide for information purposes. Because of high backpressure, it is difficult to slurry pack particles smaller than 3 μm in

long capillary columns [281]. Electrokinetic [302,311] and centrifugal [316] packing methods have been described as possible solutions to this problem, but little used. In the electrokinetic method a suspension of packing material in a buffer solution is electrokinetically migrated into an empty capillary containing a bed-retaining frit. The density and homogeneity of the column packing depends on the applied voltage and particle size distribution. The packing material should have a narrow size range to minimize particle segregation during the packing process.

The formation of bed-retaining frits is recognized as a problematic step in the column fabrication process. The bed-retaining frits must be mechanically strong to resist the pressure used to pack and/or equilibrate the column. At the same time, the frits must possess high permeability to solvent flow. The most widely used fabrication method is the thermal sintering of a portion of the column bed in the presence of water [294,302,315,317-320]. This is simply achieved using a number of commercial devices (ring heaters, optical splicers or microburners) capable of applying heat to a small area of the column. Either a high temperature ($> 550^{\circ}\text{C}$) for a few seconds or a lower temperature for a longer time (430°C for about 15 seconds) is typically used. These high fusion temperatures destroy the protective-polyimide column coating resulting in columns that are fragile at the frit position and difficult to handle. The organic portion of chemically bonded phases is also destroyed during sintering, and the sintered particles may become active, adsorbing polar analytes and causing peak tailing. Treatment of the frits with a silanizing reagent to reduce their activity and adjust electroosmotic flow is possible but inconvenient. Frit fabrication procedures lack reproducibility and sintering changes the characteristics of the packing contributing to non-uniformity of the electroosmotic flow, additional extracolumn band broadening, and bubble formation. Bubble formation occurs in the open section of the column duplex due to an increase in electroosmotic flow on going from the packed region through the frit into the open section [267,319]. Sintered frits are likely to have a zeta potential that is different from the column bed, and this discontinuity can lead to the development of flow-equalizing intersegmented pressure at the frit with concomitant bubble formation. Pressurization is widely used to reduce bubble formation. Either by pressurizing the whole column (100-200 p.s.i.) by applying pressure simultaneously to both electrolyte reservoirs or by applying pressure at the inlet end of the capillary to generate additional flow. In the later case, a parabolic flow profile reducing column efficiency results.

To ameliorate the problems associated with sintered frits alternative approaches to frit fabrication continue to be developed. Some promising methods include sol-gel-glued packing materials [321,322], thermally polymerized silicate solutions [323], monolithic frits formed by photopolymerization of a mixture of organic monomers and a porogen [293,324] and tapered joints (which allow fritless column configurations) [310,325]. The robustness of these methods remains to be explored. None are simple, but all tackle at least some of the problems associated with sintered frits, and provide a platform for further development.

8.4.2.2 Monolithic Stationary Phases

Monolithic stationary phases have emerged in the last few years as an attractive alternative to particle-packed capillary columns as a method to completely eliminate the need for bed-retaining frits and their associated problems [302,306,307]. The dimensional stability of the monolithic structures results from their rigidity and/or chemical attachment to the inner wall of the capillary. Several synthetic strategies have been described, but the most useful are based on molded porous polymers [306,326-331], molded porous sol-gel continuous beds [302], hydrothermal immobilization of packed beds [332] and particle-fixed continuous beds [302,333,334].

Molded porous polymer and sol-gel monoliths are prepared directly in the capillary column by first filling the column with a solution containing all the necessary reagents and then using heat or light to initiate the polymerization process. Individual recipes are complicated and contain several different components required to provide the desired retention properties, adequate electroosmotic flow, and a uniform and narrow-pore size distribution. Molded porous polymer monoliths are dominated by soft or rigid macroporous poly(methacrylate ester), poly(acrylamide), and to a lesser extent, poly(styrene) monoliths. A typical poly(methacrylate ester) monolith is prepared by thermal initiated free radical copolymerization of monomers (e.g. a mixture of butyl methacrylate, ethylene dimethacrylate and 2-acrylamido-2-methyl-1-propanesulfonic acid) and a free radical initiator (e.g. benzoyl peroxide or azobisisobutyronitrile) in the presence of a porogenic solvent (e.g. a mixture of water, 1-propanol and 1,4-butanediol). Inclusion of an ionic monomer and optimization of the pore size results in an adequate and stable electroosmotic flow over a wide pH range. Polymers with either ternary or quaternary amino groups (e.g. 2-dimethylamionethylmethacrylate) followed, in some instances, by a chemical modification step, are suitable for the preparation of monoliths with weak or strong anion-exchange properties [327]. Preliminary results indicate that monoliths with a small uniformly linked polymer microglobules and large canal-like pores of a few hundred nm to over 1 μm , are essential for high separation efficiency [328]. In addition to providing permeability, the pore structure reduces band broadening by increasing the rate of mass transfer within the monolithic structure as a result of convection. On an equal length basis, monolithic columns provide a similar separation efficiency and flow characteristics to particle-packed columns. The experimental conditions required to prepare monolithic columns of high separation efficiency, however, are not as well developed as slurry packing techniques.

Less information is available for sol-gel silica monoliths, which are difficult to prepare in a capillary format [302]. The preparation procedure is similar to organic polymer monoliths employing a solution containing one or several silica precursors (e.g. tetraethoxysilane), which are hydrolyzed and condensed at a defined pH in a specific solvent mixture. The sol solution first forms a hydrogel at room temperature, which is then converted to a mechanically stable and porous xerogel by thermal treatment. The xerogel is either used directly or after silanization to form a chemically bonded phase. Problems can arise from shrinking or cracking of the silica bed during

drying, from the difficulty in controlling the pore size distribution (which may be bimodal) and in homogeneously derivatizing the monolith.

At present it seems that immobilization of silica-based particles within a packed capillary by hydrothermal treatment or sol-gel adhesion represent a simpler approach to the preparation of silica-based monoliths for capillary electrochromatography [302,332-334]. Particle fixation is achieved through adhesion by silica precipitated in the interparticle space released from the particles by hydrothermal treatment, or formed by hydrolysis and polycondensation of a solution of alkoxysilanes (sol-gel process). Since only relatively low temperatures are used in both processes, chemically bonded phases can be immobilized as easily as silica. The selectivity and separation efficiency of immobilized particle beds is generally similar to that of slurry packed columns prepared from the same stationary phases.

8.4.2.3 Open Tubular Columns

Because of unfavorable mass transfer properties in liquids, highly efficient separations and short separation times potentially available for open tubular columns can be realized only in columns of small internal diameter ($< 25\ \mu\text{m}$) [309]. These columns have very low phase ratios and serious detection problems arise. Several methods have been proposed to increase the surface area, and hence the stationary phase capacity, by chemical etching of the interior wall [335] or by adhesion of a thin porous silica or polymer layer to the wall [336-338]. The sol-gel process allows an increase in surface area and formation of a retentive chemically bonded phase in a single step. None of these processes, however, adequately address the problems of low retention, low sample capacity, poor sample detectability, and unfavorable handling characteristics that prevent wider use of open tubular columns in capillary electrochromatography.

8.5 CAPILLARY GEL ELECTROPHORESIS

Capillary gel electrophoresis is used for the electrophoretic separation of biopolymers in capillaries filled with buffer and a sieving medium [11,12]. It is most suitable for the separation of polyelectrolytes with similar mass-to-charge ratios that differ by size. The development of replaceable sieving media, full automation, on-line quantification, compatibility with small sample amounts, and capillary array instrumentation have allowed capillary gel electrophoresis to compete effectively with conventional slab gel electrophoresis. It has emerged as the technique of choice for high-throughput genome sequencing [339-344] and for the analysis of antisense oligonucleotides [345] and detection of gene mutations [346]. The phosphate group of each nucleotide unit carries a negative charge that is much larger than the charge contribution from the base component above pH 7. Thus, for polynucleotides, the mass-to-charge ratio is independent of the base composition, and nearly identical for each DNA molecule.

Historically, slab or capillary gel electrophoresis was commonly used to determine protein molecular mass [347-350]. Today, MALDI-MS is rapidly becoming the method of choice for this purpose in molecular biology. Native proteins vary in charge, shape, and size making it difficult to estimate their molecular mass by electrophoresis. Denaturing under reducing conditions with sodium dodecyl sulfate (SDS) causes most proteins to assume the same globular shape and have a constant mass-to-charge ratio. Boiling a protein in a slightly basic solution ($\text{pH} \approx 8$) with 0.1 % (w/w) SDS in the presence of mercaptoethanol results in the destruction of disulfide linkages and binding of the polypeptide chains with a nearly constant amount of SDS (1.4 g SDS / g protein). The Ferguson method is used for glycoproteins and lipoproteins, which do not adsorb SDS to the same extent as other proteins. Electrophoretic separations at different gel concentrations are determined and the logarithm of the mobility of each protein is plotted against the gel concentration. The protein size is determined from the slope. Capillary gel electrophoresis has been little used for the separation of synthetic polyelectrolytes [351-353], although this remains a potential growth area.

In the above examples the purpose of the sieving medium is to provide a size-based separation, since individual polynucleotides and SDS-proteins have virtually identical size-to-charge ratios and cannot be separated by capillary zone electrophoresis. The uniformity of the polyelectrolytes allows a simple empirical relationship between size and molecular mass to be developed. The sieving matrix entangles larger polyelectrolytes and slows their migration through the capillary. In this way, smaller polyelectrolytes reach the detector first followed by progressively larger polyelectrolytes.

Two types of sieving matrices are commonly used: chemical gels represented by crosslinked polymers immobilized (usually) by wall attachment; and physical gels (or entangled polymer networks) represented by water soluble, non-crosslinked linear polymers with a flexible and dynamic pore structure [341,354-359]. Chemical gels are almost exclusively poly(acrylamide) with a pore size controlled by varying the ratio of acrylamide and the crosslinking reagent N,N'-methylenebisacrylamide. Problems that can arise in column fabrication include bubble formation due to gel shrinkage during polymerization and crosslinking of surface-bonded gels, and slow extrusion of free gels from the column induced by the electroosmotic flow. Gel extrusion can be minimized by eliminating the electroosmotic flow by covering the column wall with a thin film of poly(acrylamide) prior to polymerizing the gel monomers in the column. Once formed the pore structure cannot be varied and the high viscosity of the gels prevents replacement or hydrodynamic injection. Columns are sensitive to temperature changes and are easily damaged in a number of ways, including precipitation of samples or entrapment of particle impurities. Chemical gels provide the highest resolution of low-mass polyelectrolytes but short operational life times and handling problems have restricted their use. A new method of preparing poly(acrylamide) gels crosslinked with allyl- β -cyclodextrin promises to ameliorate some of these problems [359]. The physical gels have proven more versatile in practice. These are fully replaceable, insensitive to temperature changes (in fact temperature programming can be used to vary apparent porosity), and allow hydrodynamic sample injection. Physical gels include

linear poly(acrylamide), poly(dimethylacrylamide), alkyl and hydroxyalkylcellulose derivatives, agarose, dextran and poly(ethylene oxide) of different mass range and concentration. Denaturing agents such as urea and formamide are generally used for the separation of single stranded DNA and for DNA sequencing. Non-denaturing conditions are used to separate double stranded DNA and to detect point mutations in single stranded DNA. Coated columns are commonly used with both chemical and physical gels to minimize electroosmotic flow and interactions of the sample with the silanol groups of the column wall (section 8.2.7). Some physical gels such as poly(dimethylacrylamide) and poly(ethylene oxide) are strongly adsorbed by the capillary wall and simultaneously provide control of capillary wall properties and a sieving medium [357,358].

Polymer selection for DNA separations is arbitrary and empirical, primarily because the mechanism of DNA separation in entangled polymer solutions is not fully understood [2,340,353,360-363]. Successful working conditions have been established for the diagnostically important range of 20-500 base pairs, and for those who prefer, standardized reagents in kit form together with working instructions are available [364]. DNA molecules greater than about 20-50 kbp (kilo or thousand base pair) cannot be separated using a constant field strength, but can be separated in an alternating electric field [340,365-367]. Separations of fragments as long as several Mbp have been obtained and their motion recorded by fluorescence microscopy. These large fragments are seen to switch from an elongated linear configuration to a U-shaped configuration with a frequency that depends on the polymer concentration and other factors. Arriving at a size-based model accommodating such flexible motion through a sieving medium is complicated theoretically [363,368]. Often quoted classical models such as the Ogston model and the reptation models must be viewed as somewhat approximate and limited. In the Ogston model, a DNA molecule is assumed to migrate as a rigid spherical particle through a network of linear fibers. Its electrophoretic mobility is proportional to the volume fraction of the pores that the DNA can penetrate. The Ogston theory does not account for the fact that large DNA fragments can stretch or deform and squeeze their way through pores smaller in diameter than the effective radius of the particle. Reptation models assume that DNA travels through a matrix as a flexible chain that reptates "snake-like" through an imaginary tube in the polymer matrix. Experimental data seem to fit the "biased reptation with fluctuations" (BRF) model the best. At high field strength large DNA chains orient themselves in the field direction, assuming a stretched rod-like conformation with fluctuations in the original tube length. The BRF model predicts the major observation in capillary gel electrophoresis, that the mobility of DNA is inversely proportional to size (base number) and the presence of a plateau mobility beyond a critical size. Models based on a permanent or transient pore model have difficulty explaining the separation of DNA molecules at polymer concentrations well below those required for entanglement. "A transient entanglement coupling" mechanism has been proposed to explain these separations. It was hypothesized that DNA fragments migrating through the polymer solution entangle or collide with individual polymer molecules and are forced to drag them through the solution, resulting in a decrease in

DNA mobility. Larger DNA molecules were postulated to have a higher probability of encountering and entangling polymer molecules, and hence experience a greater reduction in mobility.

8.6 CAPILLARY ISOELECTRIC FOCUSING

Capillary isoelectric focusing (CIEF) separates amphoteric compounds in order of their isoelectric points (pI) in a steady state pH gradient maintained by an electric field [350, 369-373]. The isoelectric point of an amphoteric compound is the pH at which the compound has a net charge of zero. Synthetic compounds called ampholytes (low molecular mass polyaminocarboxylates) with different pI values are used to establish the pH gradient along the capillary [370,374]. Ampholytes have good buffering properties at their pI values and are capable of maintaining a stable steady state pH gradient. Amphoteric samples migrate in this pH gradient until they reach their isoelectric points where they become stationary and focused into narrow bands. Isoelectric focusing is used almost exclusively for the high-resolution separation of proteins and peptides in complex mixtures; for the determination of protein microheterogeneity (e.g. glycoform analysis); as a diagnostic tool in clinical chemistry (e.g. identification of hemoglobin variants and thalassemias); and for monitoring purity and degradation of protein therapeutic agents (e.g. immunoglobulins, transferrin) [350,372-376]. Capillary isoelectric focusing is also widely used to determine the isoelectric point of unidentified proteins [371,377]. There are a few applications outside of protein chemistry to small organic zwitterions and amphoteric dyes.

Typically, a fused-silica capillary column coated with poly(acrylamide), is filled with a 1-2 % (w/v) solution of carrier ampholytes. The sample is either dissolved in this solution or injected separately. A coated capillary column is used to minimize electroosmotic flow and reduce interactions between proteins and the capillary wall. An agarose gel plug, prepared with the electrolyte solution added to the electrode reservoir, may be inserted into one end of the column to prevent hydrodynamic flow in the capillary during the focusing step. The cathode and anode ends of the capillary are immersed in 20-40 mM sodium hydroxide and phosphoric acid, respectively. Strong acid (base) electrolytes are used to prevent the carrier ampholytes from migrating into the electrode reservoirs. A potential of 0.5-1 kV/cm is applied across the capillary until a steady state is attained, and the current falls to a fraction (10-25 %) of its initial value. The contents of the capillary are then mobilized by chemical or hydrodynamic means, causing the focused bands to move past the detection window. In a modification of the general approach, dynamic coating or an ionic coating is used to control the electroosmotic flow, such that focusing is complete before the sample bands arrive at the detector [378,379]. Also, whole column imaging detection has been proposed to eliminate the need for the mobilization step, but is not widely used [380,381]. Most manufacturers of capillary electrophoresis instruments support capillary isoelectric focusing by supplying capillary isoelectric focusing kits for their instruments [382].

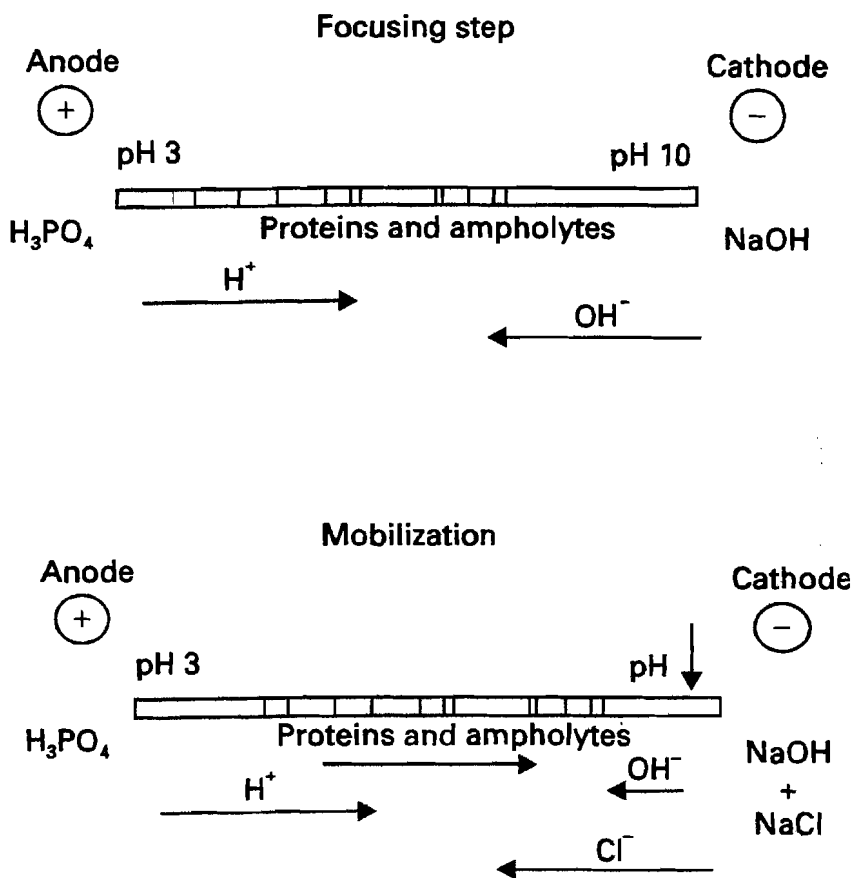


Figure 8.9. Schematic diagram of the two-step process in capillary isoelectric focusing using a coated capillary column. The top figure illustrates the steady state focussing mechanism and the bottom figure chemical mobilization by adding salt to the cathode reservoir. The focussed protein bands are transported past the detector located at the cathode end of the capillary.

Due to suppression of the electroosmotic flow, a post-separation mobilization step is required to transport the focused stack of carrier ampholytes and proteins past the detector [369,378,383,384]. The two common approaches for mobilization are chemical or hydraulic processes. The addition of a salt to one of the electrode reservoirs and re-establishment of the voltage across the capillary causes the transport of the column contents towards that electrode reservoir, Figure 8.9. This is the most widely used approach. The intrusion of migrating ions, other than hydronium or hydroxyl ions, into the focussed zones causes the pH of the zones to change as the ampholytes acquire charge and migrate in the electric field. The net result is that steady state conditions are never reached again in the capillary, and the entire gradient moves in the direction of the

detector. Hydrodynamic mobilization is performed by applying pressure at the capillary inlet, by vacuum at the capillary outlet, or by siphoning (gravity mobilization) at high electric field strengths to minimize band broadening due to the parabolic flow profile of the electrolyte solution through the column.

At or in the proximity of their pI values proteins exhibit a minimum total charge and reduced solubility in the electrolyte solution [350,370,385]. This increases the probability of aggregation, and is further enhanced by the low ionic strength of the ampholyte buffer. Under these conditions protein precipitation results from hydrophobic interactions. These interactions can be suppressed by addition of additives such as ethylene glycol (10-40 %), non-ionic or zwitterionic surfactants (1-4 %), or sorbitol to the ampholyte buffer.

High-resolution separations by capillary isoelectric focusing are favored by a shallow pH gradient, the low diffusion coefficients of proteins and high electric field strengths [350,369,371,374]. The ampholyte composition determines the range of the pH gradient and resolution within that range. Carrier ampholytes are heterogeneous mixtures of hundreds of chemical species synthesized from oligoamines by addition of α,β -unsaturated acids. A broad-range ampholyte mixture (e.g. pH 3-10) is useful for the separation of complex mixtures with a wide range of pI values, or for screening proteins with unknown pI values. For high resolution, an ampholyte mixture with a range as small as 1-pI unit can be used. Resolution within a given range is a function of the number of ampholyte species in the mixture and blends of different ampholyte mixtures are used to enhance resolution. Addition of compounds to act as spacers to locally flatten the pH gradient in the region of the analytes also improves resolution. Protein bands differing by as little as 0.01 pI can be resolved with appropriate experimental conditions. The simultaneous focussing of proteins and marker compounds, or correlating pI values with the current associated with the transit of a peak at the detector allows pI values to be determined with a precision of about 0.03 pH units [377,383].

8.7 CAPILLARY ISOTACHOPHORESIS

Capillary isotachopheresis (CITP) is used to separate ions in a discontinuous buffer system based on differences in their electrophoretic mobility [2,12,386-391]. The sample is sandwiched between two electrolyte solutions of different composition. The first solution, or leading electrolyte, contains an ion of the same sign as the sample ions with an electrophoretic mobility that is higher than that of any of the sample ions of interest. The second electrolyte solution, or terminating electrolyte, contains an ion of the same sign as the sample ions with an electrophoretic mobility less than that of the least mobile sample ion of interest. The separation is achieved by application of an electric field in the constant current mode. The sample ions are separated into a series of contiguous discrete zones with sharp boundaries containing single analytes with different electrophoretic mobilities. The potential across each zone is constant, but at the zone

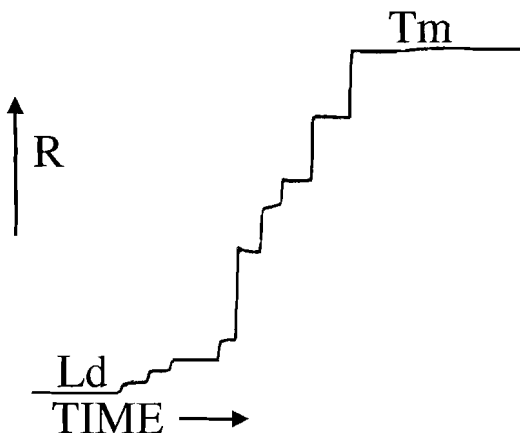


Figure 8.10. A typical capillary isotachopherogram illustrating the stepped change in the conductivity detector response as the train of stacked zones migrates passed the detector (Ld = leading electrolyte and Tm = terminating electrolyte).

boundary, the potential increases to maintain the same electron flux (current). Eventually a steady state is reached in which the stack of sample and electrolyte zones migrate at the same velocity without spaces at the zone interfaces. Detection of the zones results in a characteristic step signal with the step height related to the mobility of the ion and a step length proportional to the sample amount, Figure 8.10. Detection is usually performed with a conductivity detector and occasionally by absorption detection.

Capillary isotachopheresis was well established before the introduction of capillary electrophoresis, but was quickly overshadowed by the rapid development of the latter. Current use is limited for analytical applications [387-389] with capillary electrophoresis being preferred in most cases. Renewed interest in capillary isotachopheresis as a sample concentration and preseparation technique for capillary electrophoresis is responsible for a somewhat limited revival. The self-sharpening and concentration characteristics of capillary isotachopheresis make it more suitable than capillary electrophoresis for preparative scale separations, where single run and continuous flow instruments have been described for the isolation of milligram to gram quantities of material [392-394]. Capillary isotachopheresis is also suitable for the determination of values for effective ion mobility [395,396].

The stepwise migration of ions under the influence of an electric field is an important feature of capillary isotachopheresis. Should an ion diffuse out of its zone, it will experience the electric field, which is driving the zone into which it has diffused. This will cause it to either accelerate or decelerate back into its own zone, depending on the magnitude of the electric field. This effect produces a self-sharpened zone, which serves to maintain sharp zone boundaries.

Electrolyte solutions and operating conditions for capillary isotachopheresis are selected based on a knowledge of sample properties, Table 8.8 [12,386,397]. The

Table 8.8

Composition of some common capillary isotachopheresis buffers

Ammediol = 2-amino-2-methyl-1,3-propanediol; HPMC = hydroxypropylmethylcellulose; and OAc = acetate

Property	pH 2.0	3.3	4.5	6.0	8.8
Separation	cations	anions	cations	anions	anions
Leading ion	10 mM HCl	10 mM HCl	10 mM KOAc	10 mM HCl	10 mM HCl
Leading counterion		β -Alanine	HOAc	Histidine	Ammediol
Leading additive		0.2 % HPMC		0.2 % HPMC	0.2 % HPMC
Terminating ion	10 mM TRIS	10 mM capr- oic acid	10 mM HOAc	10 mM MES	10 mM β - Alanine
Terminating counterion	HCl			Tris TRIS	Ba(OH) ₂
Terminating pH	8.5			6.0	9.0
<i>Recommendations</i>					
Leading ion (20-30 mM)	Cations K^+ , NH_4^+ , Na^+			Anions Cl^-	
Terminating ion	H^+ , or weak base (mobility $> H^+$)			OH^- , or weak acid (mobility $> OH^-$)	
Terminating counterion	Weak acid, $pK = pH_L \pm 0.5$			Weak base, $pK = pH_L \pm 0.5$	
Typical counterions	pH_L			pH_L	
Formate	3.2-4.2			β -Alanine	
Acetate	4.2-5.2			Histidine	
MES	5.7-6.7			Imidazole	
Glycine	9.1-10.1			TRIS	
				Ethanolamine	
				9.0-10	

leading ion is required to have the same sign and a higher mobility than the sample ions. The counterion is generally a weak acid or base capable of providing sufficient buffering capacity at the desired pH for the leading electrolyte. The terminating ion is usually hydrogen ions for cations (or hydroxide ions for anions). Analyte ion mobilities are modified by adjusting the pH of the separation medium or by adding complex forming reagents to the leading electrolyte and sample. Nonionic and zwitterionic surfactants or urea can be used to adjust solubility. Separations are generally performed in the absence of electroosmotic flow using chemically bonded or dynamically coated [e.g. poly(ethylene glycol), hydroxypropylmethylcellulose, etc.] fused silica (< 0.2 mm I.D.), or wide bore PTFE (0.5-0.8 mm I.D.), capillary columns. The electroosmotic velocity depends on the choice of the leading and terminating electrolytes as well as the sample composition. Moreover it changes continuously during the separation, and is the principal cause of poor reproducibility of migration times, and poor quantitative precision. On the other hand, the presence of electroosmotic flow facilitates applications employing bidirectional capillary isothachopheresis for the simultaneous separation of anions and cations. In this case, the leading electrolyte for the separation of cations must also be the terminating electrolyte for the anions, and *vice versa*. The anions migrate in the opposite direction to the cations, but are carried towards the cathode by the electroosmotic flow with a velocity less than that of the cations.

It follows from the principle of the separation process, that the sample ion concentration changes during the separation until it is adjusted to a constant value. This value depends on the concentration of the leading electrolyte, and the mobility of the sample ion, leading ion, and common counterion. Another important consequence of the separation mechanism is the concentration effect. A sample ion more concentrated in the original sample than the steady state concentration is diluted during the separation, while an ion that is more dilute is concentrated. The adjusted concentration of analytes in their zones is constant for a given electrolyte system, no matter whether a diluted or concentrated sample is injected. Hence, the length (volume) of the adjusted zone is proportional to the sample amount. The length of the detector cell sets the detection limit for conductivity detection. Zones shorter than this length (e.g. < 0.2 mm) can be separated but are not registered by the detector.

The on-line coupling of capillary isotachopheresis in two dimensions (CITP-CITP), or more commonly with capillary electrophoresis (CITP-CE, CITP-CITP-CE), combines the advantages of capillary isotachopheresis for sample concentration and matrix simplification with the greater flexibility and detection options for capillary electrophoresis [390,391,398-401]. A general limitation of single-column capillary electrophoresis is unsatisfactory concentration detection limits owing to the small injection volumes, and short path length flow cells of absorption detectors. In addition, sample matrix components may adsorb onto the capillary wall affecting the electroosmotic flow, causing poor reproducibility of migration times, and perhaps rendering the column useless for further separations. Using capillary isothachopheresis as the first and independent separation mode, sample concentrations can be amplified by 10^3 - 10^4 fold, and highly concentrated components diluted and swept away from the second column. Wide-bore capillary columns allow sample volumes of 10s to 100s of μl to be injected. Flow diversion, controlled by independent electrodes with different voltages, allows concentrated fractions from the first column to be selectively transferred to the second column for separation. A detector at the column interface can be used to identify the desired transfer zones. In the CITP-CE mode, the isothachopheresis capillary is filled with the leading electrolyte and the electrophoresis capillary with the terminating or some other background electrolyte. After some time, the background ions penetrate through the whole system, and all zones are destacked and migrate in the electrophoresis mode. For now the general use of CITP-CE and CITP-CITP-CE, is limited by availability of suitable instruments, and the need for a better understanding of the influence of the transition from isotachopheretic to electrophoretic separation conditions in the second capillary on the quality of the electrophoretic separation.

8.8 METHOD DEVELOPMENT

A general guide to method selection based on established common applications is provided in Table 8.9. Reasonable solubility in aqueous solution is required for

Table 8.9

Common applications of capillary electromigration techniques

Technique	Separation mechanism	Applications
CE	Differences in charge-to-size ratios	Inorganic and organic ions Ionizable compounds Zwitterions Biopolymers
MEKC and MEEKC	Distribution of neutral and partially ionized compounds between charged micelles (or emulsion) and the electrolyte solution	Water soluble neutral compounds Weak acids and bases
CEC	Distribution between a solid stationary phase and mobile electrolyte solution	Neutral compounds Weak acids and bases Ions
CGE	Differences in size and charge (but not size-to-charge ratio) by migration through a gel matrix or entangled polymer network with a range of pore sizes	DNA fragments SDS proteins Macromolecules
CIEF	Differences in isoelectric points in a continuous pH gradient	Proteins Zwitterionic compounds
CITP	Differences in electrophoretic mobility of ions sandwiched between two buffers containing ions of greater (leading) and lower (trailing) mobility	Preconcentration of ions

most separation modes. Nonaqueous capillary electrophoresis is little developed, and although micellar electrokinetic chromatography can separate hydrophobic compounds, selectivity is usually limited. Liquid chromatography is often a better choice for these samples and when low level detection or preparative-scale separations are required. The concentration sensitivity of capillary-electromigration separation techniques using absorption detectors is limited by the small cross-column path length and small injection volumes to solutions containing at least 1–10 $\mu\text{g/ml}$, and for ease of operation, 0.1 mg/ml or higher. Various stacking and preconcentration techniques (sections 8.9.3) are available to improve detection limits, but these require additional effort and time for optimization, and are not easy to justify if another technique is suitable for the separation.

Samples should be prepared such that the analytes of interest are present in a suitable solution, free from interferences, and at an appropriate concentration for detection. The ionic strength of the sample should be no greater than that of the buffer, with a more or less similar pH to the buffer, and free of matrix problems associated with column wall adsorbing materials and particle matter. For the best peak shapes and resolution, the concentration of the injected sample should be about 100 times lower

Table 8.10

Guide for selecting initial conditions for capillary-electromigration separation techniques

Parameter	Setting
Column	Initial experiments use a fused-silica capillary 30-50 cm long and 50- or 75- μm I.D. Short columns are appropriate for scouting experiments. The complexity of the sample dictates the required column length. For 2-10 analytes use 35-40 cm; 11-50 analytes 50-60 cm; 50-80 analytes 70-80 cm; and > 80 analytes 90-100 cm. Smaller diameter columns (25 or 50 μm) provide higher separation efficiency but lower sample loading capacity.
Initial column conditioning	Rinse with 1 M sodium hydroxide for 15 min. Flush with water for 10 min followed by the separation buffer for 10 min.
Voltage	Usual range is 10–30 kV. High voltages provide faster separations and higher separation efficiency. The method employed to dissipate heat, the column internal diameter, and buffer type and concentration all affect this decision. Use the highest voltage that does not exceed 100 μA current (or the highest current recommended for the instrument) as a rough guide for CE and MEKC. Otherwise plot current against voltage (2.5 kV increments) and operate at a voltage towards the upper portion of the linear plot.
Temperature	Initial experiments use 20-25°C. Selectivity and separation speed varies with temperature, which is optimized to fine-tune a separation (vary from 20 to 60°C in 5°C increments).
Injection	Hydrodynamic (e.g. 3 s at 0.5 p.s.i.) or electrokinetic (2-5 nl)
Detection	Absorption maximum of the analyte of interest, for which the weakest signal is expected because of low concentration or low absorbance. If analyte detection properties are unknown try 200-230 nm.

than the buffer concentration. Syringe filters for particle removal, and ion exchange membrane filtration devices to reduce excessive concentrations of common matrix ions, are available. Proteins and similar macromolecules, if not of interest to the analysis, should be precipitated before separation to minimize column fouling. Analytes of low water solubility may have to be dissolved in a water miscible organic solvent or mixture of organic solvent and separation buffer. For other samples it is common practice to dissolve the sample in the run buffer, a diluted solution of the electrolyte solution used for the separation, or water.

8.8.1 System Variables

Virtually all separations are carried out in fused-silica capillary columns of 50-100 μm internal diameter up to 1 m in length, Table 8.10 [402-404]. Wide-bore capillary columns provide greater loading capacity and a higher detector response because of the longer path length (on-column detection), but generate higher currents and provide lower separation efficiency due to less efficient heat dissipation. If detection limits are not a problem, then a small diameter column should be used. The choice of length

is a compromise between speed (short columns) and peak capacity (long columns). Unless the separation is unusually complicated, capillaries should be short (25-50 cm). When a new capillary is put into service, or is suspected of being contaminated, a conditioning procedure is required (section 8.9.1). Columns with a chemically bonded or physically adsorbed interior coating are used to control electroosmotic flow, or to minimize analyte adsorption by the capillary wall, particularly for macromolecules (section 8.2.7). Electroosmotic flow is optimized to obtain useful separations in micellar electrokinetic chromatography and capillary electrochromatography, but is usually undesirable for capillary gel electrophoresis, capillary isoelectric focusing, and capillary isotachopheresis.

8.8.2 System Optimization

8.8.2.1 Capillary Electrophoresis

Once the system variables are set within reasonable ranges, the parameters that have most effect on migration times and selectivity are the composition, concentration and pH of the electrolyte solution, and the presence of additives, if used, to provide additional selectivity [100,101,404-406]. Four features are important for an acceptable separation: (i) the analytes must have different mobilities; (ii) the electrolyte solution must be homogeneous and the field strength uniform along the column; (iii) neither analytes nor matrix components must interact with the column wall; and (iv) the conductivity of the electrolyte solution must exceed the conductivity of the sample. Suitable buffers with their pK_a and ion mobility values are given in Table 8.2.

Ionic strength and pH greatly affect selectivity and separation time and should be course tuned in initial screening experiments. Low pH is favorable for separating anions (all anions are less mobile) and a high pH is preferred for separating cations. The practical pH range is limited roughly to between 2 and 12. If the pK_a of the sample components is known, or can be reasonably estimated, pH optimization should start with a $pH \approx pK_a$. Weak acids and bases change from the neutral form to the fully ionized form over about 4 pH units. Partially ionized analytes migrate with an effective mobility that changes in a sigmoid fashion, as the pH is varied (see Figure 8.1). Ions may be separated in their fully ionized form, or partial ionized form, as a matter of circumstance; that is, at those conditions that maximizes the difference in charge-to-size ratios. Because changes in mobility tend to be large for partially ionized solutes, small pH changes (0.1-0.5 pH units, or smaller for complex mixtures) are used to optimize separations.

If the pK_a values for a sample are unknown, initial separations in a series of buffers at or near pH 2.5, 4.0, 5.5, 7.0, 8.5, and 10 are conducted. From the plot of the effective mobility against pH, the most promising pH range for the separation can be identified. Optimization then proceeds by smaller pH changes, as before.

To optimize the buffer concentration, initial experiments are performed with a concentration of 30-100 mM for 50- μm internal diameter columns and 20-50 mM with 75- μm internal diameter columns. Lower ionic strength buffers are used to obtain

Table 8.11

Secondary chemical equilibria used to optimize selectivity in capillary electrophoresis

Additives	Function
Inorganic salts	Minimize wall interactions, induce protein conformation changes
Crown ethers	Modify mobility by selective formation of inclusion complexes
Organic solvents	Modify electroosmotic flow, increase solubility of organic ions, modify ion solvation, reduce wall interactions
Urea	Modifies the mobility of proteins by hydrogen-bond complexation
Metal ions	Modify mobility of anions and electroosmotic flow
Alkanesulfonic acids	Modify mobility by ion pair formation, wall adsorption leads to changes in surface properties
Cellulose polymers	Mask active sites on the capillary wall, modify electroosmotic flow
Cationic surfactants	Use to reverse the direction of electroosmotic flow
Organic acids	Modify mobility by ion pair formation
Chelate formation (metals)	Polycarboxylic acids (lactate, tartrate, hydroxyisobutyric acid) Ethylene-1,2-diaminetetraacetic acid Dihydroxyazobenzene-5,5'-disulfonate
Ion pairing	Ionic surfactants (< critical micelle concentration) Cetyltrimethylammonium bromide, tetradecyltrimethylammonium bromide Polyvalent metal cations (Ca^{2+} , Al^{3+} , etc.) CHES and other alkanesulfonic acids, perchlorate
Ion inclusion	Crown ethers (15-crown-6, 18-crown-6, etc.) Cyclodextrins

faster separations, when selectivity is high, and to separate simple mixtures containing a few analytes. Higher ionic strength buffers are required for the separation of complex mixtures, or to separate analytes with small differences in their electrophoretic mobility. If stacking is used to enhance analyte detectability, then the difference in ionic strength between the electrolyte solution (high ionic strength) and the sample should be maximized. Inorganic buffers are likely to provide better peak shapes for high mobility ions and zwitterionic buffers for low mobility ions. Zwitterionic buffers are useful for many applications where a high concentration and buffering capacity is desirable because of their low specific conductivity, which allows more favorable kinetic separation conditions to be employed.

For difficult separations, the selectivity can be further modified by employing secondary chemical equilibria, and solvation effects by adding appropriate reagents or solvents to the electrolyte system, Table 8.11 [404]. Increasing the ionic strength

of the electrolyte by adding salts, such as potassium sulfate, modifies the charge and/or conformation of proteins and reduces wall interactions [68,350,407]. In addition, alkanesulfonic acids bind selectively to proteins and peptides through hydrophobic interactions, modifying the surface charge as well as reducing wall interactions. Slow adsorption/desorption interactions with the column wall cause peak broadening and tailing, and irreversible adsorption leads to modification of the capillary wall. These problems are caused by electrostatic or hydrophobic interactions between macromolecules (usually) and the column wall. Solutions to this problem include: using extreme pH buffers; high ionic strength electrolytes; and by using dynamic or chemically bonded wall coated columns (section 8.2.7). There is no universal solution, and effective methods have to be tailored to the properties of the analyte. Buffer additives are typically used at concentrations of 5-60 mM, except for modification of the ionic strength of the electrolyte solution, where much higher concentrations are often required (e.g. 50–250 mM). Urea, which forms hydrogen-bond complexes with proteins and peptides, but is non-ionic, is often used at concentrations of 7 M. A large number of additives have been described to aid the separation of amino acids and biogenic amines [408]. Carbohydrates are separated in their anionic form at high pH, as charged borate complexes, and after derivatization with charge-bearing groups [409-411]. The separation of metal cations (alkaline earths, transition metals and lanthanides) is difficult because of their similar equivalent conductance [54,412-414]. Complexing agents, such as α -hydroxyisobutyric acid, lactic acid or citrate are generally required for their separation, Figure 8.11 [415]. Since many cations lack a chromophore, complexation is an effective method of introducing a chromophore for convenient detection. The fast separation of anions requires the reversal of the electroosmotic flow by adding cationic surfactants below their critical micelle concentration to the electrolyte solution [405,406,416-419]. The electroosmotic flow and electrophoretic migration now occur in the same direction. For difficult separations, normal (counter flow) operation may be a better option at the expense of longer separation times. To reduce peak broadening the mobility of the sample anions should be matched to those of the electrolyte solution. For UV-visible detection, indirect detection is frequently employed (section 8.9.5.3). This requires the addition of a visualizing ion of high molar absorption, at low concentration, and of the same charge as the analytes. Examples include chromate (most popular), benzoate, salicylate and phthalate.

8.8.2.2 *Micellar Electrokinetic Chromatography*

An acceptable separation requires favorable kinetic properties (efficiency), provision of an adequate migration window (peak capacity), adequate selectivity, and a reasonable total separation time. Normally, the experimental conditions are set to establish an acceptable separation time and migration window, under conditions where the separation efficiency is not compromised, and the outcome of the experiment controlled by selectivity optimization [158,201,404]. Selectivity is influenced largely by the identity of the surfactant and the addition of complexing agents and/or organic solvents to the buffer (section 8.3.3). Method development usually begins with sodium dodecyl

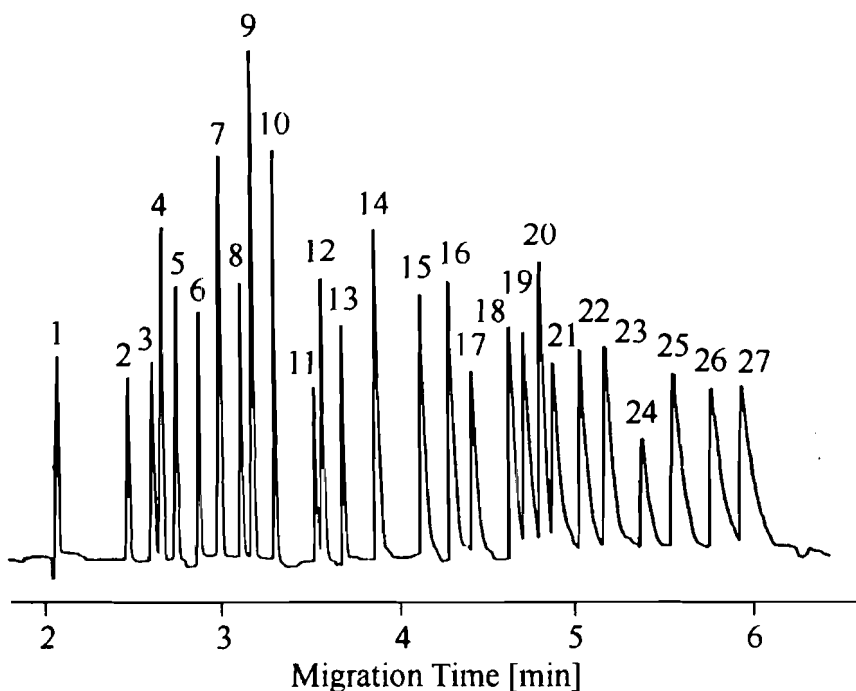


Figure 8.11. Separation of metals by capillary electrophoresis on a 60 cm x 75 μ m I.D. fused-silica capillary column at 20°C with a potential of 30 kV. The electrolyte solution contained 15 mM lactic acid, 8 mM 4-methylbenzylamine and 5 % (v/v) methanol at pH 4.25. (From ref. [415]; ©Elsevier).

sulfate because of its favorable kinetic and chromatographic properties, Table 8.12. Other surfactants are selected based on their complementary properties to sodium dodecyl sulfate using the system constants of the solvation parameter model as a guide (Table 8.5). A working list of surfactants for selectivity optimization would include sodium dodecyl sulfate, sodium cholate, lithium perfluorooctanesulfonate, sodium N-dodeconyl-N-methyltaurine, and tetradecyltrimethylammonium bromide. Surfactant concentration changes the phase ratio, but has little effect on selectivity

When selectivity optimization using different surfactant types is exhausted, further optimization is achieved by use of additives. For this purpose, the common approaches are the use of mixed surfactant micelles, organic solvents, and inclusion complexing agents. A large number of mixed micelles can be employed without any certain prospects of success (section 8.3.3.1). Neutral surfactants, such as Brij 35, are often chosen first to adjust selectivity and/or the size of the migration window. Selectivity modification by addition of organic solvent to the buffer is by no means as useful as in reversed-phase liquid chromatography. At low concentrations, modifier effects are small and not strongly dependent on solvent identity, and at higher concentrations, they lead to deleterious effects on system efficiency and the separation time. By contrast,

Table 8.12

Starting conditions for method development in micellar electrokinetic chromatography

Parameter	Setting
Sample	1-2 mg/ml dissolved in methanol or water
Column	Fused-silica capillary 30-50 cm long with 50 μ m I.D.
Initial conditioning	Flush with 0.1 M sodium hydroxide for 3 min and rinse with the run buffer for 5 min. These conditions will have to be varied depending on the previous use (if any) of the column. It is preferable to reserve individual columns for each surfactant.
Buffer	20 mM sodium phosphate-sodium tetraborate pH 8 buffer containing 50 mM sodium dodecyl sulfate
Voltage	20-25 kV
Temperature	25°C
Injection	50 mbar 2-4 s (hydrodynamic)
Detection	210 nm (or absorption maximum for analyte with lowest absorbance)

Course tuning selectivity

Surfactant	Choose surfactants of different selectivity (see Table 8.5) Sodium cholate (75 mM) Sodium N-Dodecanoyl-N-methyltaurine (50 mM) Tetradecyltrimethylammonium bromide (50 mM) with reverse polarity Other suitable surfactants
pH	Optimize migration window and separation time (lower pH to extend and raise pH to lower) for neutral compounds. Weak acids and bases may show significant changes in electrophoretic behavior.
Additives	Mixed surfactants formed with neutral and ionic surfactants. For example, Brij 35 (polyoxyethylene [23] dodecyl ether) 1-25 mM Organic solvents methanol, 2-propanol, acetonitrile, tetrahydrofuran 1-25 % (v/v) Higher molecular mass solvents of low water solubility 1-5 % (v/v) Complexing additives such as α -, β -, γ -cyclodextrins, hydroxypropyl- β cyclodextrin and heptakis-(2,3,6-tri-O-methyl)- β -cyclodextrin (5-20 mM)

the use of complexing additives, such as urea and cyclodextrins, has to be considered one of the success stories of micellar electrokinetic chromatography for achieving the separation of isomers, enantiomers, and other difficult to separate compounds capable of forming suitable inclusion complexes (section 8.3.6).

8.8.2.3 Capillary Gel Electrophoresis

Capillary gel electrophoresis is used for the separation of macromolecules such as proteins and nucleic acids, whose mass-to-charge ratios do not vary much with size

[340,341,350,373,404]. Separation requires a sieving medium made up of a crosslinked gel or an entangled polymer network. The capillaries are often wall coated or chemically bonded to minimize electroosmotic flow that tends to destabilize the columns. Columns filled with rigid crosslinked gels, usually poly(acrylamide), are characterized by the total amount of monomer and crosslinking agent (%T), and the ratio of crosslinking agent to total amount of monomer and crosslinking agent (%C), used to prepare the column. Larger pore size gels (lower %T) are used for separating DNA sequencing reaction products, whereas narrow-pore media are used for the separation of proteins and small oligonucleotides. Entangled polymer networks of linear poly(acrylamide), methylcellulose, or dextran have the advantage that they can be forced into the capillary as a solution, and replaced for each separation. Unlike gels, these columns are easily prepared in the laboratory and tend to be more robust. Electrokinetic injection is used for sample introduction. The buffer pH is selected such that the analytes of interest are ionized. TRIS/borate and TRIS/phosphate buffers, pH 7.5-8.5 and 50-200 mM are commonly used. Sometimes urea (7-8 M) or ethylene glycol (1.5-3.0 M) is added to the buffer as a nonionic denaturing or solubilizing agent, and EDTA (about 2 mM) to protect against cation interferences. When SDS-proteins are separated, sodium dodecyl sulfate (0.1 % w/v) is added to the run buffer. For many practical applications of capillary gel electrophoresis, the column materials and reagents required can be purchased in kit form.

8.8.2.4 *Capillary Isoelectric Focusing*

Capillary isoelectric focusing is used to separate polypeptides based on differences in their isoelectric points (pI), in coated columns, to eliminate electroosmotic flow and non-specific adsorption of the sample with the capillary wall. The capillary is filled with the sample and a mixture of ampholytes capable of producing a pH gradient covering the pI range of the proteins. In practice about 94% of proteins can be separated in the pH range 3-8.5. When a voltage is applied (e.g. 15 kV for 4 min), the sample components focus into narrow zones according to their pI values. The zones are then mobilized by hydraulic, electroosmotic or ion addition (by adding 80 mM sodium chloride to either the source or destination vial and applying an electric field) to move them passed the detector. The destination vial contains a buffer (catholyte) at a pH higher than the pI of the most basic ampholyte (40 mM sodium hydroxide) and the source vial contains a buffer (anolyte) at a pH lower than the pI of the most acidic ampholyte (20 mM phosphoric acid). To avoid protein precipitation in the focused zones a surfactant or urea can be added to the buffer, the sample diluted, or gel filled capillaries can be used.

8.9 INSTRUMENTAL ASPECTS

Modern instruments for capillary-electromigration separation techniques are characterized by a high level of automation, computer control and safety features [8-13, 420-422].

These have largely replaced self-assembled systems [423] and provide improved performance and ease of use. General features include: a capillary column for the separation; a high voltage power supply to drive the separation; an automated sample introduction system for hydrodynamic or electrokinetic injection (section 8.9.2); a detector to determine the presence and amount of individual analytes (section 8.9.5); and a computer with appropriate software for instrument control, and recording and integration of the electropherogram. Additional features might include a thermostat for column temperature control, and a pneumatic module for pressure-assisted separations. A solvent-management system for gradient elution, and a system to maintain a positive and equal pressure at the inlet and outlet end of the separation column, to minimize bubble formation, may be installed for capillary electrochromatography (section 8.9.4).

A high voltage source is a prerequisite for capillary-electromigration separation techniques. Commercial instruments are limited by electrical breakdown of insulators and the atmosphere to a maximum of about 30 kV with currents of 200 μ A, although instruments operating at higher voltages have been described [123,424,425]. The separation efficiency and separation time are directly proportional to the applied field strength, explaining the interest in ultrahigh voltage operation, but as well as mechanical problems, column temperature gradients must be minimized by effective thermal management. In addition to the absolute voltage, the power supply must be capable of high precision ($\pm 0.1\%$) and low drift. Contemporary instruments all provide for operation at constant voltage, with some capable of constant current and constant power operation as well. Constant current operation provides compensation for temperature fluctuations and improved intra-day precision for migration times, while constant voltage, which is more common, provides better inter-day precision. The power supply should have the capability to switch polarity. This is best accomplished using a dual polarity power supply grounded at the detector side of the capillary, and fitted with a ground-leakage detector for protection. In operation, the high voltage electrode is driven either positive or negative with respect to the ground electrode. Field programming is a useful feature offered by software control. It allows the voltage to be rapidly changed at the start of a separation to avoid rapid heating of the buffer and expulsion of sample from the capillary. It also facilitates heartcutting and fraction collection, in support of advanced separation techniques.

Thermostating the column allows active temperature control. Thermostating ($\pm 0.1^\circ\text{C}$) is important for obtaining reproducible migration times [13,426,427]. Absolute temperature has only a minor effect on the separation efficiency, although radial temperature gradients are responsible for establishing the maximum field strength for the separation (section 8.2.3). Different thermal-management systems are used to realize capillary thermostating. These are based on circulating air or liquid and Peltier coolers. Liquid cooling is considered superior to forced air convection and allows column operation with higher power dissipation but is awkward to implement. With cassette type column holders, the capillary ends may not be temperature controlled, and some detector configurations require the capillary to be extended outside the thermostat, opposing the benefits of accurate temperature control. Temperature programming

is a practical and useful method for shortening the separation time and optimizing the separation factor for capillary electrochromatography [428], but is not useful for other capillary-electromigration separation techniques.

8.9.1 Columns

Ideally, the separation column should be chemically and electrically inert, (preferably) transparent to UV light, flexible, robust, and inexpensive. These requirements are met to a reasonable extent by fused-silica capillary columns with a protective polyimide covering. Columns of this type, in various lengths up to about 1 m and 25-100 μm internal diameter dominate the practice of capillary-electromigration separation techniques. Similar columns packed with 3-5 μm diameter particles are used for capillary electrochromatography (section 8.4.2) and filled with a polymeric gel or entangled polymer solution for capillary gel electrophoresis (section 8.5). The electroosmotic flow and undesirable analyte interactions with the column wall can be modified by either dynamic or permanent wall coatings (section 8.2.7) as required for specific applications. Swelling of the polyimide coating of fused-silica capillary columns in acetonitrile-containing buffers was identified as the reason for several problems (poor separation performance, clogging of the capillary ends, break off of column ends) [429]. The long-term stability of the columns was improved by heating to 300°C before use. A number of different organic polymer hollow fibers have been used in capillary isotachopheresis for many years, but have found only limited application in other capillary-electromigration separation techniques [430-432]. When non-transparent fibers are used, a small section of fused-silica capillary column as a detection window can be inserted between two pieces of the fiber, by means of PTFE sleeves. The main problems with polymeric fibers is poor control of the capillary internal diameter, poor heat transfer properties, and an adsorptive character, which although different to fused silica, is still detrimental to separation performance for some applications.

Most instruments use a cassette design to position the separation capillary in the thermostat and to connect the column with the buffer reservoirs and detector. Columns can be purchased with a detection window located at the correct position for insertion into the cassette. Alternatively, a number of different types of ring heaters, optical splicers and microburners are available for those who wish to prepare their own columns at a lower cost [294,315,317]. A detector window is prepared by vaporizing a small section of the polyimide coating to leave a transparent section of fused silica about 1-2 mm long at a fixed distance from the column outlet. Once the window is formed, the column is fragile, and must be handled carefully. It is also important that the column ends are cut square and free of debris since misshapen column ends can result in distorted peak shapes and poor quantitative precision.

The optimization and reproducibility of the surface chemistry of fused silica is an important property that directly affects the stability of the electroosmotic flow, the reproducibility of migration times, and the adsorptive properties of the column

wall [144,426,433-436]. The requirement that the column supplies the mechanism for controlling the electroosmotic flow and produces the separation at the same time, is a weak point of capillary-electromigration separation techniques. The separation quality depends on the column history, and poorly understood properties of the sample matrix and buffer composition. Some form of column conditioning is essential to obtain reproducible separations when a new column is first placed into service, and a periodic conditioning step is often required between separations for acceptable repeatability. However, there is little science to the selection of optimum conditions for column conditioning, since this process varies widely with sample properties, and the quality and composition of the electrolyte solution. Column conditioning is generally performed by rinsing the capillary with some combination of 0.1 to 1.0 M sodium hydroxide, 1.0 M hydrochloric acid, water and perhaps a water-miscible organic solvent. Sequential treatments are easily automated with an autosampler that allows the use of overpressure to force the conditioning solutions through the column for a specified time. One function of the rinse step is to clean the column wall of adsorbed material from prior samples or the electrolyte solution. The optimum solvent or sequence of solvents in this case will depend on the type of material adsorbed onto the wall. A second function is to restore the chemistry of the capillary surface to its former value by maintaining a constant concentration of ionized silanol groups and surface-attracted counterions. In all cases, the last step in column conditioning is a rinse step with the separation buffer. When possible it is good practice to dedicate individual columns for specific separations, since it can be difficult to obtain repeatable results with a column used for a different set of separation conditions. This applies particularly to separations by micellar electrokinetic chromatography using different surfactants and just about any separation system where specific additives are incorporated in the background electrolyte. When designing the conditioning sequence, it is advisable to avoid extreme changes in pH, which can result in slow equilibration and surface hysteresis effects. A further consideration is that elaborate conditioning procedures, or unnecessary conditioning steps, significantly reduce sample throughput.

8.9.2 Sample Introduction

Columns used for capillary-electromigration separation techniques have volumes no greater than a few microliters. If band broadening is to be minimized then extracolumn volumes (injection and detection) should be no more than about 1 % of the column volume. Normal injection volumes, therefore, are restricted to no more than a few nanoliters. Managing such small injection volumes is not particularly difficult with fully automated hydrostatic, hydrodynamic, or electrokinetic sample introduction methods [8,12,13,423,427]. The modest method precision when compared with valve injection (of generally larger sample volumes) in liquid chromatography, however, tells another story. Several undesirable effects, such as siphoning, ubiquitous injection, and sample carryover are known to affect accuracy of electrophoretic methods.

Siphoning is caused by the pressure difference across the separation column arising from differences in the liquid level of the electrolyte reservoirs or formation of droplets

at the column ends during exposure of the column end to air. It results in variable injection volumes and band broadening during the separation. Siphoning is minimized by leveling the electrolyte solution in the destination and origination vials, and by using narrow capillary columns and rapid transfer of the column inlet between the sample vial and the electrolyte reservoir for the start of the separation. Ubiquitous injection results from a number of events that occur at the column ends driven by the interfacial forces that are generated at solution interfaces. Droplet formation, siphoning, movement of the sample on the outside of the capillary, and evaporation are possible factors at the air-liquid interface affecting the injection volume. If the separation capillary remains in contact with the sample solution for more than a few seconds then additional sample loading may occur as analyte diffuses into the capillary. The outside surface of the capillary and the electrode may cause sample carryover and contamination of sample and buffer solutions. Carryover is minimized by removing of the polyimide coating at the tip of the separation capillary and frequent renewal of electrolyte and conditioning solutions.

8.9.2.1 Hydrodynamic Injection

Hydrodynamic injection methods are based on the creation of a pressure difference across the separation column with the column inlet located in the sample vial by any of three general methods: raising the height of the sample vial above the level of the column outlet; by overpressure of the sample vial; by application of a vacuum to the detector end of the capillary. The average length of the sample zone, L_{inj} , introduced is given by

$$L_{inj} = d_c^2 \Delta P t_{inj} / 32\eta L \quad (8.30)$$

where d_c is the column internal diameter, ΔP the pressure difference across the column (for hydrostatic injection $\Delta P = \rho g \Delta h$ where ρ is the density of the electrolyte solution, g the gravitational force constant, and Δh the height difference between the column ends), t_{inj} the injection time, η the viscosity of the electrolyte solution filling the column, and L the column length. The sample solution flows into the capillary with a parabolic flow profile and the sample zone is spread out over a distance $2L_{inj}$. From the plate number required for the separation, a maximum value for L_{inj} is easily calculated, $L_{inj} < 2L / \sqrt{N}$, and used to calculate a value for the product $\Delta P t_{inj}$ in Eq. (8.30). For optimum precision the column temperature should be constant (L_{inj} depends on η), short injection times should be avoided (e.g. 1-2 s), and an internal standard should be used. In favorable cases, a precision $< 2\%$ RSD is possible, but it is nearly impossible to obtain values of L_{inj} sufficiently small to have no influence on the separation efficiency without compromising precision. Hydrodynamic injection using overpressure of the sample vial is generally more precise and robust than electrokinetic injection, but may not be applicable when packed columns are used for the separation. Contemporary instruments automate the injection process, allowing sequential analysis and intermediate-column conditioning, in conjunction with a carousel or 96 well plate autosampler.

8.9.2.2 Electrokinetic Injection

For electrokinetic injection a low voltage is applied for a brief time with the inlet of the separation column located in the sample vial. Sample enters the column by the combined effect of electroosmosis and electrophoresis. If it is assumed that the conductivity of the sample solution and background electrolyte is equal, then the length of the injected zone, L_{inj} , is given by

$$L_{inj} = (\mu_{eo} + \mu_{ep})V_{inj}t_{inj} / L \quad (8.31)$$

where V_{inj} is the voltage applied during the sampling step. The sample zone entering the capillary is plug like, but since mobilities are signed quantities an analyte with a mobility in the same direction as the electroosmotic flow occupies a longer zone than one migrating in the opposite direction. The electrophoretic component of the injection process results in discrimination with the more mobile sample components injected in larger quantities than the less mobile species. Changes in ionic strength and matrix composition of the sample solution affect its conductivity, altering both the electrophoretic and electroosmotic flow rates and the absolute amount of sample introduced into the column. Whenever possible, samples should be prepared at a low concentration in the separation buffer, to avoid this problem. Band broadening effects are possible whenever substantial differences in pH of the sample and separation electrolyte exist. As current flows through the sample solution during injection, electroactive species may be altered in composition. Electrokinetic injection is the common form of injection for capillary electrochromatography and capillary gel electrophoresis, but for other methods hydrodynamic injection is preferred.

8.9.3 Preconcentration Techniques

Capillary-electromigration separation techniques provide good mass detection limits, which are advantageous when the sample volume is limited, such as for the analysis of single cells. Concentration detection limits are generally more important for samples that are not volume limited, and in this respect, capillary-electromigration separation techniques compare poorly with liquid chromatography. Poor concentration sensitivity is a consequence of the limited sample injection volume and the short across column path length of photometric detectors. Precolumn or in-column concentration techniques, based on electrokinetic and chromatographic methods, go some way to mitigating this problem by allowing larger sample amounts to be loaded onto the separation column [437-442]. Besides these techniques, liquid-liquid extraction [443] and microdialysis sampling probes [437,441,444] have been used for sample enrichment and cleanup for specific sampling problems. Microdialysis probes, for example, are widely used for the *in vivo* sampling of biological fluids and the extracellular environment of living organisms. Capillary isotachopheresis (section 8.7) is widely used for sample concentration and cleanup in single column or multidimensional separation modes together with capillary electrophoresis [445,446].

Table 8.13

Rough guide to the enhancement factor for different preconcentration techniques

Technique	Enhancement factor	Detection limits	Reproducibility (RSD %)
Field-Amplified Sample Stacking (FASS)	1000	80 nM	3-25
Large-Volume Sample Stacking (LVSS)	100	50 nM	5-12
pH-Mediated Stacking	10-80	0.3-30 μ M	2-10
Capillary Isotachopheresis (CITP)	10-10,000	10-100 nM	0.3-3
Sweeping	80-1,000,000	0.1 nM	3-15
Chromatographic Preconcentration	500-7000	5 nM	3-6

8.9.3.1 Electrokinetic Methods

Electrokinetic methods of preconcentration are based on the difference in electrophoretic velocity in the sample solution and electrolyte solution for the separation when a voltage is applied during or after injection. The sample ions are concentrated or stacked at the boundary between the sample and electrolyte solutions as they decelerate from a region of high field strength to one of low field strength induced by a change in conductivity of the two solutions. The increased signal-to-noise ratio of the peaks in the electropherogram (enhancement factor) is a result of the reduction in peak width, caused by the deceleration focusing mechanism and an increase in the sample amount loaded onto the column. The reduced width of the focused sample zone allows larger sample volumes to be injected without loss of separation efficiency. Enhancement factors vary tremendously with the experimental conditions, and some approximate values are summarized in Table 8.13 [438,439,442].

Field-amplified sample stacking (FASS) is the most straightforward of the in-column preconcentration techniques [12,13,159,438,439,447-449]. A long plug of sample in a low conductivity solution is introduced by hydrodynamic injection into the separation column filled with an electrolyte solution of higher conductivity. The column inlet is returned to the electrolyte solution for the separation and a high voltage applied. Due to the difference in conductivity of the two solutions, the field strength is higher over the sample zone, and falls to a low value over the electrolyte zones. The high electrophoretic velocity of the analytes in the sample zone comes to a near stop as they enter the electrolyte solution due to the sharp decline in field strength. Consequently, sample ions become concentrated in a narrow band at the boundary between the sample and electrolyte solutions. The ultimate enhancement factor is limited by the difference in conductivity between the sample and electrolyte solutions and the sample volume that can be injected into the separation column. The maximum sample volume is limited by a decrease in separation efficiency caused by laminar flow within the column, owing to the mismatch between the local electroosmotic velocity and the bulk velocity. The flow profile resulting from laminar flow is parabolic and creates band broadening in the narrow sample zone. Large concentration differences between the sample and electrolyte solution result in a larger enhancement factor, but also greater laminar flow. Optimum experimental conditions are a compromise, therefore, between maximizing the enhancement factor and minimizing band broadening. A difference

in conductivity between the sample and electrolyte solutions of about an order of magnitude is considered optimum.

Higher enhancement factors are possible in FASS if the sample is loaded by electrokinetic injection. In this case, analytes are injected by electromigration, and those ions with an electrophoretic velocity higher than the electroosmotic velocity, are loaded at a higher concentration. The low conductivity of the sample zone ensures high field strength, so sample ions migrate rapidly into the capillary and stack at the interface between the sample and electrolyte solutions, due to the sharp decline in field strength at the boundary. A disadvantage of the electromigration injection method is that the amount of sample ions introduced into the separation column depends on the sample conductivity and the ion electrophoretic velocity. Since the stacked sample ions have an electrophoretic velocity that is less than the electroosmotic velocity, it is possible for them to be moved out of the separation column if they migrate in the direction opposite to the electroosmotic flow. A solution to this problem is to inject a low conductivity or water plug into the separation column, immediately in front of the sample. This short plug provides a zone of high field strength at the injection point. The analyte ions will then migrate rapidly into the separation column and stack at the other end of the plug, regardless of the direction of electroosmotic flow. Field amplification can also be used for enrichment of analyte ions migrating in the opposite direction to the electroosmotic flow in combination with polarity switching. Hydrodynamic injection methods are generally more reproducible and do not discriminate against ions of lower mobility.

Large-volume sample stacking (LVSS) allows an increase in the sample volume introduced into the column without loss of separation efficiency, Figure 8.12 [12,438,439,449,450]. This requires that the sample matrix is removed from the column after the stacking process, to eliminate the nonuniform distribution of both the field strength and the electroosmotic velocity, before the electrophoretic separation begins. Up to 60-90 % of the separation column is filled with a low conductivity sample solution by hydrodynamic injection (the rest of the column is filled with a higher conductivity electrolyte solution). A reverse potential is applied to the column, causing electroosmotic flow towards the injection end of the column. The electroosmotic flow backs the sample solution out of the column, while the negatively charged sample ions migrate towards the detector, and stack at the boundary between the sample and electrolyte solutions. An essential requirement of this method is that the analyte ions have a higher electrophoretic mobility than the electroosmotic mobility of the sample solution. As the sample solution is removed, the separation column is gradually filled with the higher conductivity electrolyte solution from the detector end of the column with the progress indicated by an increase in the operating current. When the current reaches a normal value, indicating that the separation column is nearly filled with electrolyte solution, the polarity is switched to the normal configuration for the separation step. Careful monitoring of the current is required to avoid expulsion of analyte ions from the separation column. To stack and separate positive ions, the charge on the column wall

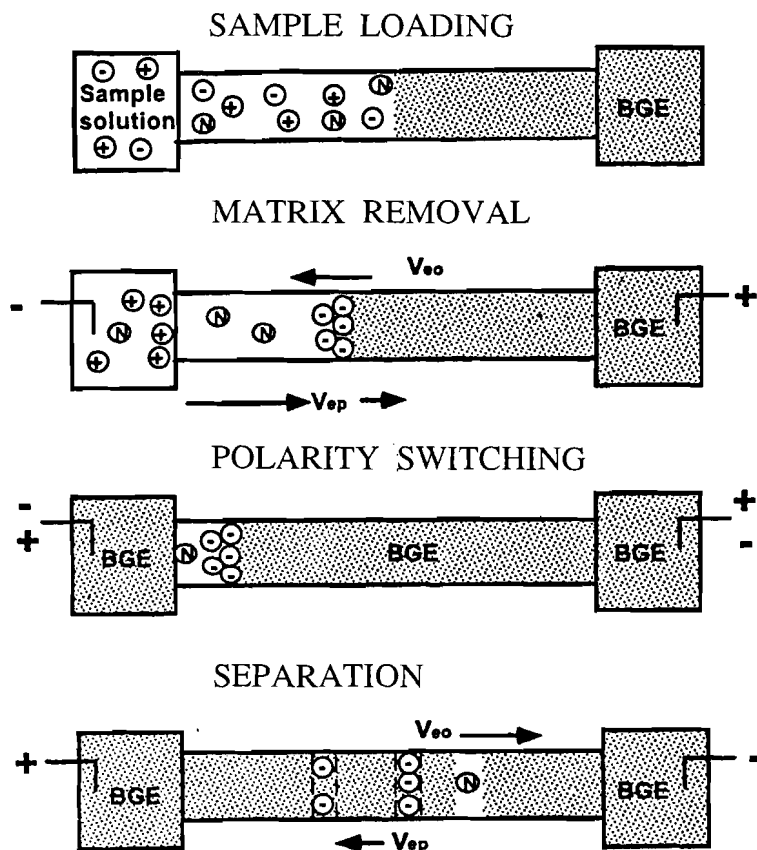


Figure 8.12. Preconcentration of negative ions by large-volume sample stacking. N represents neutral sample components. (From ref. [12]; ©Wiley-Interscience).

has to be changed by adding a quaternary ammonium salt to the electrolyte solution to reverses the direction of the electroosmotic flow.

Field-amplified and large-volume sample stacking is performed with the sample either dissolved in water or diluted with a low conductivity electrolyte solution. These conditions may be unsuitable for concentrating analyte ions in a high ionic strength sample matrix. A solution to this problem is pH-mediated sample stacking using electromigration titration to neutralize the sample matrix [438,439,451]. Analytes are injected electrokinetically in an electrolyte solution containing a low-mobility weak-base (such as ammonium). During the injection phase, cations in the sample (such as sodium) are gradually replaced by the weak-base cation. Completion of sample injection is followed by electrokinetic injection of a hydroxide solution of similar ionic strength to the electrolyte solution. The hydroxide ions migrating into the separation column titrate

the ammonium ions in the sample zone to form a low conductivity neutral zone that gradually extends through the column. Anions in the sample zone, therefore, experience high field strength and migrate to the boundary between the sample and electrolyte solutions, where they are stacked as a result of the lower electric field strength. While this method is a welcome advance for the analysis of high ionic strength samples, the capillary column volume ultimately limits the extent of preconcentration unless a coupled column approach is used. Strict control over the concentration and injection time of both the sample and the titrating zone is also required to ensure reproducible results.

High salt and sweeping methods are used for stacking neutral analytes for separation by micellar electrokinetic chromatography [159,452-458]. Simply adding a salt to the sample solution to raise its conductivity above that of the separation electrolyte in an electric field results in stacking of the charged micelles at the detector side of the sample solution. Neutral analytes carried out of the sample zone with the electroosmotic flow are efficiently concentrated at the micelle front because of their high concentration at the boundary zone. Sweeping is the process of accumulating neutral and charged analytes by surfactant micelles that penetrate the micelle-free sample zone driven by an applied potential. The sample solution need not be prepared in a low ionic strength matrix, but similar conductivity to that of the electrolyte solution is favored. The separation column is initially filled with an electrolyte solution containing surfactant micelles. The sample in water or electrolyte solution without micelles is injected hydrodynamically to provide a sample zone of the desired volume. The column inlet is then placed in the electrolyte solution and a voltage applied to drive the micelles through the sample solution, sweeping the neutral analytes along with them, where they stack at the boundary between the region of high micelle concentration and the sample solution. The use of either cationic or anionic micelles, suppression of the electroosmotic flow, and normal or reverse polarity allows conditions to be established for the concentration of neutral and charged analytes.

8.9.3.2 Chromatographic Methods

Chromatographic preconcentration methods can be used for both enrichment and matrix simplification [437,439,441,459-463]. Chromatographic methods have the advantage that the sample volume is independent of the separation column volume, allowing larger injection volumes to be used. It is possible to combine chromatographic and electrokinetic preconcentration techniques to further increase the enhancement factor. A typical setup for on-line solid-phase extraction and capillary electrophoresis consists of a capillary preconcentration column directly attached to the separation column by a PTFE sleeve, Figure 8.13. The preconcentration column contains a few milligrams of a typical liquid chromatographic microparticle sorbent, sandwiched between two glass fiber frits in a packed bed a few millimeters long, or in the form of a particle-loaded membrane disk. Typical sorbents include non-selective porous polymer or silica-based chemically bonded phases, or selective immunoaffinity, molecularly imprinted polymers, and ion-exchange sorbents. The sample is loaded onto the precolumn by

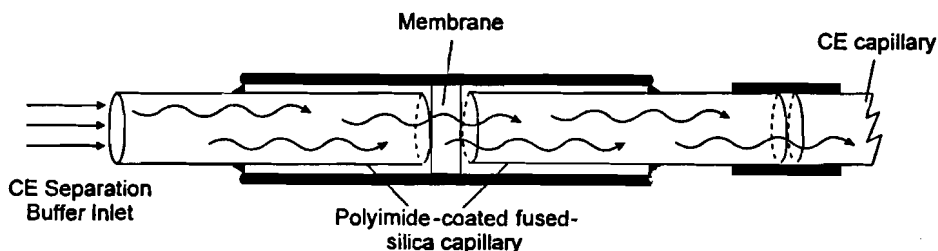


Figure 8.13. Preconcentration cartridge for on-line solid-phase extraction and capillary electrophoresis.

hydrodynamic injection and undesirable matrix components removed by a cleanup step, usually by rinsing with the same electrolyte solution selected for the electrophoretic separation. The retained analytes are eluted from the column with a small plug of solvent (e.g. 50-100 nl of buffer containing an organic solvent). The presence of organic solvent aids sample stacking at the start of the electrophoretic separation. Problems include blockage of the separation column or extraction precolumn by precipitated material or particles; the limited choice of electrolyte solutions, which must be compatible with the extraction sorbent; the need for a small volume of desorption solvent; and peak tailing or reduced separation efficiency that compromise separation performance. Chromatographic preconcentration techniques are effective but require substantial effort to establish robust operating conditions, and are not widely used on a routine basis. Conventional off-line solid-phase extraction is a convenient alternative for routine laboratories when the available sample volume is not restricted. Multidimensional and comprehensive coupled-column systems combining reversed-phase liquid chromatography and capillary electrophoresis provides an additional option for the analysis of complex mixtures (section 5.6.2).

8.9.4 Pressure-Assisted and Gradient Elution Separations

Instruments capable of hydrodynamic injection can be used for pressure-assisted separations with open tubular columns if designed so that the inlet reservoir can be sealed after insertion of the separation capillary and high-voltage electrode. Overpressure of the inlet reservoir imparts a pressure-driven flow to supplement the electroosmotic flow through the separation column. This results in shorter separation times at the expense of lower separation efficiency. It can be used with any separation conditions, but is particularly useful for those conditions that result in weak electroosmotic flow.

Higher pressures are required for pressure-assisted separations in packed columns used for capillary electrochromatography [235,376,464-466]. These columns require a high-pressure pump to provide the supplementary mobile phase flow. Conventional rotary injection valves and autosamplers can be used for sample introduction with this arrangement if a special inlet tee housing the electrode and split line with a restrictor is installed [422]. Microcolumn pumps are also useful for conditioning columns before initial use in capillary electrochromatography (section 8.4.2). There are no com-

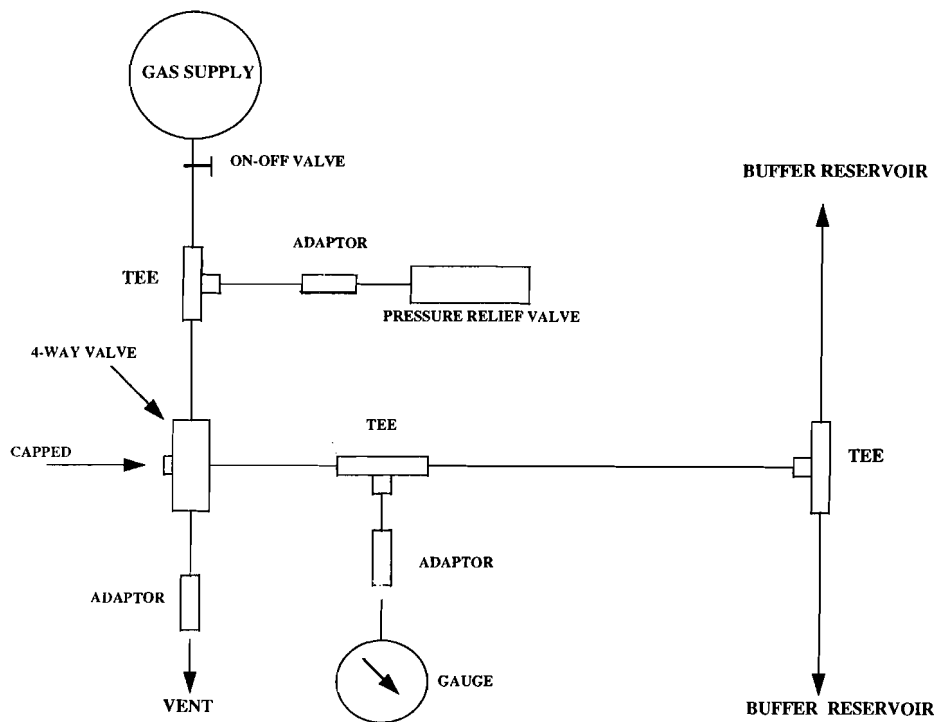


Figure 8.14. Schematic diagram of a pneumatic system retrofitted to a capillary electrophoresis instrument for capillary electrochromatography with simultaneous pressurization of the column inlet and outlet reservoirs.

mercial instruments available for capillary electrochromatography with supplementary pressure-driven flow. The expected advantages are a stable and reproducible column flow rate, independent of the electroosmotic flow properties of the column, at the expense of reduced separation efficiency due to the parabolic flow profile of the pressure-driven flow component.

Packed columns with sintered frits (section 8.4.2) are generally operated at an elevated pressure to minimize bubble formation by applying an equal pressure to the column inlet and outlet electrolyte solution reservoirs. Pressures less than 500 p.s.i. are adequate for this purpose. A suitable setup to adapt a capillary electrophoresis instrument for use in capillary electrochromatography with pressure-equalized column operation is shown in Figure 8.14 [262].

Gradient elution separations in capillary electrochromatography are desirable for all the same reasons that make it a common separation technique in liquid chromatography (section 1.4.5). The characteristic feature of gradient elution separations is the programmed increase in mobile phase solvent strength during the separation. The simplest approach to gradient elution separations in capillary electrochromatography is

the step gradient technique, where the separation is carried out with different inlet vials containing electrolyte solutions of increasing elution strength [466]. The separation is started with a weak mobile phase, sometime later the voltage is set to zero and the weak mobile phase replaced with a stronger mobile phase and the voltage restored. This process can be repeated several times, if desired, to generate a complex gradient. Step gradients are easily automated and do not require modification to standard instruments. Instruments for continuous gradient elution are not commercially available at present, although a number of systems have been described based on the transfer of preformed gradients to the separation column [262,264,422,466-471], or electroosmotic pumping from two reservoirs containing different mobile phase compositions [264,466,472]. In the first case, the mobile phase gradient is prepared by a liquid chromatographic pump in the usual way and moved through a tee-piece interface and out to waste. Simultaneously, a potential is applied across the interface, electroosmotically introducing a portion of the preformed gradient into the separation column. For electroosmotic pumping, the gradient delivery system consists of two independent high-voltage power supplies controlled by a computer. Gradient mixing is performed in a tee-piece, which connects two independent mobile phase reservoirs to the separation column. This experimental setup requires three electrodes, two of them are placed in the mobile phase reservoirs, and the third is placed in the column outlet reservoir. The amount of each solvent reaching the mixing tee is controlled by the independent voltage ramps applied to each reservoir. The main disadvantages of this approach are that the exact composition profile of the mobile phase is unknown and sample injection is awkward (the separation capillary must be disconnected from the tee piece and then reconnected for each injection). The mobile phase flow from each reservoir depends on the dielectric constant and viscosity of the solutions. This results in simultaneous changes in solvent composition and flow that are difficult to model.

8.9.5 Detectors

Capillary-electromigration separation techniques are characterized by excellent mass but poor concentration detection limits, Table 8.14 [8-13,422,473-476]. These features are a result of the small peak volumes and their associated constrained injection and detection volumes. Concentration detection limits are generally inferior to those obtained for liquid chromatography using similar detection mechanisms and the range of detection options is not as great. Almost all capillary electrophoresis instruments are fitted with a UV-visible absorption detector as standard, with fluorescence and conductivity detectors and interfaces to mass spectrometry as possible options. The operating principles of absorption (section 5.7.1.1), fluorescence (section 5.7.1.2) and electrochemical detectors (section 5.7.4) are similar to detectors used for liquid chromatography, and therefore, in the following sections emphasis is placed on their adaptation for efficient operation in capillary electrophoresis. Other detection modes such as refractive index [477,478], chemiluminescence [479-483], light-scattering [484], and radiochemical detection are possible with various levels of difficulty using laboratory-assembled devices.

Table 8.14

Rough guide to detection limits for capillary electromigration techniques

Detection mode	Typical detection limits	
	Mass (mole)	Concentration (M)
Absorption (UV-visible)	$10^{-13} - 10^{-16}$	$10^{-3} - 10^{-8}$
Indirect		$10^{-5} - 10^{-7}$
Fluorescence	$10^{-15} - 10^{-17}$	$10^{-5} - 10^{-9}$
Laser Induced	$10^{-18} - 10^{-21}$	$10^{-10} - 10^{-16}$
Indirect	$10^{-15} - 10^{-17}$	$10^{-5} - 10^{-7}$
Chemiluminescence	$10^{-13} - 10^{-17}$	$10^{-9} - 10^{-10}$
Conductivity	$10^{-15} - 10^{-16}$	$10^{-4} - 10^{-8}$
Amperometry	$10^{-18} - 10^{-20}$	$10^{-5} - 10^{-8}$
Potentiometry		$10^{-7} - 10^{-8}$
Refractive Index		$\approx 10^{-6}$
Radiochemical		$10^{-9} - 10^{-11}$
Mass Spectrometry	$10^{-16} - 10^{-17}$	$10^{-8} - 10^{-9}$
Nuclear Magnetic Resonance		$\approx 10^{-3}$

8.9.5.1 Absorption

Absorption detectors designed for liquid chromatography generally require modification to their optical layout to improve the focusing of light into the detector cell, and to improve the effective collection of transmitted light [474,476,484-489]. The source beam should be focused to a width narrower than the inner diameter of the detection cell. The most widely used designs incorporate a sapphire ball lens with microscope objectives for this purpose. Conventional scanning detectors are too slow to cope with the short residence times of the analyte peaks in the detector cell. A diffraction grating and either imaging onto a photodiode array or rapidly scanning across a single photodiode is suitable for single or multiple wavelength detection and full spectral recording, although generally at low resolution. A fast-data acquisition system is also essential to handle peak widths of typically 1-2 s.

On-column detection is commonly used to avoid band broadening and is facilitated by the high transparency of fused silica in the low-wavelength UV region. In on-column detection, the separation capillary acts as its own cylindrical detection cell [12,13,490]. The internal diameter of the capillary and curvature of the capillary wall limit the effective path length of the cell. Light rays further away from the central axis of the capillary traverse decreasingly shorter distances until eventually approaching zero at the column wall. The effective optical path length for the absorption signal is, therefore, less than the capillary internal diameter, which is already constrained to small values ($< 150 \mu\text{m}$) by Joule heating of the electrolyte solution. These short-effective path lengths result in a relatively weak absorption signal. Scattered light at the circular capillary wall also limits the dynamic range of the detector. A number of extended path length detector cells based on a rectangular capillary [491], bubble cell [422,440,491-494], or Z-cell [422,440,494] have been proposed to enhance the detector signal. To create a bubble cell the capillary diameter is expanded by a factor of

3 to 5 in front of the source aperture. The Z-cell allows axial illumination of a portion of the separation column, or separate detection cell, in a pre-aligned cartridge holder. This is achieved by creating a Z-bend in a fused-silica capillary with a transverse path length of 1.2 mm. In these designs, the increase in detector response is a compromise between the longer path length of the cell and reduction in light throughput owing to smaller cell apertures. Typical response enhancements are 3-5 fold for the bubble cell, and 3-20 fold for the Z-cell. The bubble cell has little influence on the separation efficiency, but some loss is observed for the Z-cell. In-column detection, referring to detection across a packed segment of the separation column, provides an alternative approach to on-column detection in capillary electrochromatography [422,495]. In-column detection results in both a higher signal and higher background noise from scattered light, such that in most cases the increase in signal-to-noise is only modest, and on-column detection remains the favored option. For fluorescence measurements, detection limits were usually slightly poorer when in-column detection was used [496].

8.9.5.2 Fluorescence

Fluorescence, and especially laser-induced fluorescence (LIF), is the most sensitive detection technique for capillary electrophoresis. But since fluorescence is not a general property of most samples, it often has to be combined with precolumn or postcolumn derivatization techniques to extend its application range [11-13,474,485,497-499]. Arc lamps with filters or a grating provide flexibility in wavelength selection, but their spatial instability can be a problem in focusing the source energy into a small capillary volume. Without optimization of the optical system, detection limits can be disappointing and not much different from absorption values. Lasers have a number of advantages in this respect: the collimated beam is easily focused into a small volume; excitation power can be varied over a wide range; and the source is inherently monochromatic. On the other hand, lasers are more expensive than arc lamps, have a shorter operating life, and provide only a limited selection of operating wavelengths, particularly in the low-UV region. Lasers suitable for LIF and their excitation wavelengths include the Argon ion laser (457, 488 and 514 nm), He-Cd laser (325, 354 and 442 nm), and He-Ne laser (543.5 nm). Many optical arrangements have been proposed for LIF detection [11,425,435,447,449]. A popular design uses a fluorescence microscope objective and coaxial illumination and collection of the fluorescence signal. In this system, the laser beam is reflected from a dichroic filter and focused onto the capillary. Fluorescence is collected by the same optics, transmitted through the dichroic filter, spectrally filtered, and detected with a photomultiplier. Using the same optics for illumination and collection results in a small depth of focus and high rejection of scattered light from the capillary wall. In addition to high sensitivity, this configuration is easily adapted to sequential detection of separations in a capillary array (section 8.9.6). The ability to identify fluorescent-labeled bases for DNA sequencing has provided much of the impetus for developing wavelength resolved LIF capillary electrophoresis instruments [500].

The on-column detection configuration is the most widely used for fluorescence measurements. Higher rejection of scattered light and improved detection limits are possible with a sheath flow cell installed at the end of the separation capillary. The sheath flow cell consists of a square fused silica chamber with thin walls. A flowing stream of refractive index matching buffer flows past the separation column outlet and constrains the eluent to the core region of the stream where detection occurs. The excitation laser beam is focused, using a microscope objective, into the core stream just downstream from the column outlet. The fluorescence signal is collected at right angles with a microscope objective, spectrally filtered to reduce Raman and Rayleigh scatter, and passed through an eyepiece fitted with a pinhole to reduce the field of view of the photomultiplier to the illuminated core stream. This design allows for extremely low-mass detection limits.

8.9.5.3 Indirect Detection

The desire for a universal and sensitive detector for capillary electrophoresis is only partly met by absorption detectors. Many common inorganic and organic ions have virtually no useful absorption properties, while capillary electrophoresis provides a useful method for their separation. These circumstances have tended to dictate a more favorable reception for indirect detection in capillary electrophoresis compared with liquid chromatography (section 4.3.3.2), where a wider range of detection options are available [12,13,501-507]. Indirect detection requires the addition of a UV absorbing or fluorescent co-ion, called a probe or visualizing ion, with the same charge as the analytes, to the background electrolyte. Detection is accomplished by displacement of the co-ion by sample ions from each sample zone leading to a quantifiable decrease in the detector signal with respect to the steady state background signal. The indirect detection method is universal and does not require any additional instrumentation beyond that used for direct detection. Detector noise and drift may be increased and the linearity degraded to some extent by the presence of the visualizing ion compared with a non-absorbing electrolyte solution. This will vary with the detector design and concentration of the visualizing ion. The concentration detection limit C_{LOD} is dependent on the concentration of the visualizing ion, the transfer ratio, and the dynamic reserve defined earlier (section 4.3.3.2). Increasing the molar absorptivity of the visualizing ion simultaneously increases the dynamic reserve while allowing a reduction in the concentration of the visualizing ion, both factors contributing to a more favorable concentration detection limit. However, because of electromigration dispersion (section 8.2.5.1) the visualizing ion must have a similar mobility to the analytes for the full benefit of the high molar absorptivity to be realized. The C_{LOD} is inversely proportional to the transfer ratio (the number of moles of the visualizing ion displaced by one mole of analyte ions) defined for capillary electrophoresis by Eq. (8.32)

$$TR = (z_B / z_A)(\mu_A / \mu_B)([\mu_B + \mu_C] / [\mu_A + \mu_C]) \quad (8.32)$$

where z represents the formal charge and μ the electrophoretic mobility for the visualizing ion A, sample ion B and the counterion C of opposite charge, respectively. The transfer ratio is seen to depend on the mobility of all ions in the electrolyte solution. In order to increase the transfer ratio of the sample ions, and simultaneously decrease electromigration dispersion of the sample zones, the mobility of the co-ion should be larger than or equal to that of the sample ions. Most electrolyte systems designed for indirect detection consist of a single visualizing ion because under these conditions the displacement process is relatively simple and well understood. Using several visualizing ions can be useful to achieve better mobility matching of sample ions with one of the visualizing ions when the sample ions cover a wide mobility range. These systems are difficult to model, and further problems can arise from the propagation of system peaks that might interfere in the separation (section 8.2.5.1) [504,505].

The composition of the electrolyte solution is of paramount importance for the success of indirect detection methods and requires consideration of several factors, for example, the identity of the visualizing ion, the relative mobility of the visualizing ion, the use of an electroosmotic flow modifier (if required), and the buffer type. Buffering of the electrolyte solution is essential for reproducible and rugged separations, with unbuffered electrolytes being subject to pH changes occurring in the electrolyte reservoirs. There are four possible ways to buffer the electrolyte solution for the separation of anions: use a weak acid as both visualizing ion and buffer (e.g. benzoate and phthalate); use a counterion with buffering properties (e.g. tris[hydroxymethyl]aminomethane, triethanolamine); use a co-anionic buffer having the same sign as the visualizing ion and analytes (e.g. acetic acid, borate, phosphate); or use an ampholyte buffer having a net charge of zero at its pI value (e.g. histidine, glutamic acid). Weak acids are useful buffers for the separation of ions of low and intermediate mobility but their buffering capacity is limited by the low concentration of the visualizing ion, and is restricted to a narrow pH range ($\text{pK}_a \pm 1$). The buffering capacity of the counterion is also limited by the concentration of the visualizing ion. Co-anionic buffers have good buffering capacity but increase the number of anions in the background electrolyte, with the production of potentially interfering system peaks and the probability of reduced detection sensitivity. The latter is due to the fact that the analytes can now displace either the visualizing ion (leading to an indirect detection signal) or the added buffer co-anion (leading to no detection signal). Co-anionic buffers should be used only when there is a significant difference between the mobility of the visualizing ion and the co-anionic buffer, and the analytes have a similar mobility to the visualizing ion. Ampholyte buffers provide an almost ideal solution, but are limited to a few pH values. Optimization of separation conditions for cations is subject to similar regulation except that the role of the anions and cations in the electrolyte is reversed [502,503].

8.9.5.4 *Electrochemical*

Electrochemical detection based on conductometry, amperometry or potentiometry is used to only a limited extent in capillary electrophoresis, with only conductivity detectors commercially available [13,475,508-511]. Conductivity is a universal method for

the detection of ions. Amperometry is a selective method for detection of electroactive species by oxidation or reduction at an electrode surface (e.g. catecholamines, amino acids, carbohydrates). Potentiometry is a near-specific method for characteristic ions. In all cases, the high voltage applied to the separation capillary can lead to serious interference with the electrochemical signal. For this reason, on-column detection is rarely used. A wall-jet configuration with the sensing electrode positioned close to the outlet of the separation column (end-column detection) is the preferred arrangement. The short gap between the column outlet and sensing electrode serves to decouple the detector signal from the influence of the separation voltage without inducing significant band broadening.

The detection of the current generated by reaction at the surface of (usually) carbon fiber or copper microelectrodes at a fixed voltage is capable of low detection limits for electroactive compounds using amperometry, Table 8.14. Several approaches that allow the full possibilities of multiple electrode and pulsed amperometric detection (established techniques in liquid chromatography (section 5.7.4)) have been proven for capillary electrophoresis [508,511]. These methods are not widely used, possibly due to a lack of commercial products and support. Potentiometric detection with polymer-coated wire microelectrodes containing relatively non-specific ion exchange ionophores was used for the detection of low-mass anions or cations [510,511].

Conductivity detection has an historic home among the capillary-electromigration separation techniques having been used for many years as a standard detector in capillary isotachopheresis (section 8.7). Conductivity detectors for capillary electrophoresis and electrochromatography are based on one of three basic designs: detectors with a galvanic contact of the sensing electrodes with the electrolyte solution; systems employing suppressed conductivity detection; and contactless conductivity detection [475,509,512-519]. Measurement of conductivity by direct contact of the sensing electrodes with the electrolyte solution can be performed using electrodes that are placed in holes through the capillary wall, or better still, in an end-column, wall-jet configuration. In the latter case, the end of the separation column is housed in a stainless steel coupling connector, which centers the separation column and ensures a fixed distance between the end of the column and the sensing electrode. A platinum wire-sensing electrode is precisely aligned in the connector and is insulated from the surrounding steel body, which serves as a concentric counter-electrode. To suppress interfering redox reactions at the electrode surfaces measurements are typically made with AC voltages at a frequency of about 1 kHz.

For suppressed conductivity detection, the end of the separation column is connected to a tubular ion exchange membrane suppressor surrounded by a reservoir of regenerant solution [512,513]. The electrodes for conductivity detection are located in a separate capillary downstream of the suppressor. The high voltage electrode for the separation is located in the regenerant reservoir. In this way, the detector is decoupled from the electric field for the separation, and the electroosmotic flow generated in the separation column is used to drive the electrolyte solution through the suppressor and detector. The function of the suppressor (see section 5.7.4.1) is to neutralize electrolyte solution ions,

leading to an order of magnitude or more decrease in the background signal, and an increase in the relative response to sample ions.

At high frequency AC voltages (e.g. 40-200 kHz) it is possible to sense changes in conductivity without direct contact between the detector electrodes and the electrolyte solution [514-519]. The contactless conductivity detector consists of two tubular electrodes (0.2-2 cm long and 0.4 mm I.D.) placed over the separation column, and separated from each other by a small gap (e.g. 0.2-2 mm). Each electrode serves as a cylindrical capacitor with the solution inside the capillary being the corresponding internal wire. The gap between the electrodes acts as a resistor, responding to differences in the conductivity of the migrating zone. Conventional conductivity detectors currently provide a higher response than contactless detectors, but further improvements in design could change this situation. This, together with the advantages of operational robustness and a small effective detector volume, could result in the contactless conductivity detector acquiring a higher profile for ion detection.

The general principles of conductivity detection were presented earlier (section 5.7.4.1). The detector signal is governed by the difference in mobility of the sample ion and electrolyte co-ions [517]. Therefore, electrolyte solutions with low-mobility ions of the same charge as the sample ions provide a more favorable detector response, but are likely to promote band broadening by electromigration dispersion. In order to limit dispersion effects and maintain high detector sensitivity, amphoteric electrolytes with low equivalent conductivity at relatively high concentrations are used. This approach is limited to electrolyte solutions with pH values between 5 and 9. High concentrations of hydronium or hydroxide ions increase the background conductivity and reduce the detector response to sample ions.

8.9.6 Capillary Array Instruments

Capillary arrays provide an increase in sample throughput by exploiting the possibilities offered by parallel sample processing. Planar electromigration methods have always supported parallel sample processing by dividing the layer into parallel lanes, with each lane reserved for a different sample. Although capillary-electromigration separation methods are intrinsically faster than planar methods as measured by the separation time, sample throughput is often no better or even lower because of the throughput limitation of sequential separation methods. These problems were recognized early in genomic sequencing and lead to the development of multiplex instruments for the simultaneous separation of DNA fragments in arrays containing 96 identical separation columns (section 8.5) [520-525]. All these instruments employ laser-induced fluorescence detection because of its high sensitivity, as well as to accommodate base calling using fluorescent-labeled DNA primers for the sequencing reaction. At present the scope of these instruments is restricted to DNA sequencing, but prototype instruments with absorbance detection for screening combinatorial libraries and DNA sequencing have been described [526,527].

The capillary array DNA sequencers developed so far are all similar as far as separation aspects are concerned. Simultaneous electrokinetic injection is used to

load the capillary array from a multiwell plate containing samples. A similar multiwell plate acts as a reservoir for electrolyte solution for the separation. The process of rinsing and refilling each capillary with electrolyte solution containing the entangled polymer separation matrix before each separation sequence is programmed automatically. All detection schemes consist of a laser as an excitation source, an emission collection system, a means of discriminating different labels, and the detector itself. Confocal designs provide high sensitivity by using a high-numerical aperture microscope objective for illuminating the detection zone of the capillary and to collect the fluorescence emission. To record the separation in each capillary, a scanning system is required. Either the capillary array or source must be moved rapidly so that each capillary is sampled at a rate (≈ 2 Hz) that does not result in any loss of separation information from any of the capillaries. A system of four photomultiplier tubes, filters and dichroic beam splitters is used to separate and record signals from the four different fluorescent labels. An alternative approach uses axial illumination of the capillary array immersed in a refractive index-matching liquid and a lens at right angles to the laser beam to focus the fluorescence emission onto a CCD camera able to perform multicolor detection. Still another approach uses postcolumn detection with liquid sheath flow. Briefly, the capillary bundle is aligned in a plane inside a quartz cuvette and the column effluent confined by a secondary flow of liquid surrounding the capillary bundle. A laser beam is directed across all flow streams and simultaneously excites fluorescence from each capillary, which is collected perpendicular to the laser beam, dispersed by a diffraction grating, and imaged onto a CCD camera. Since these systems use similar separation technology, they provide roughly the same throughput of about 0.5 Mbp per day. The instruments and approaches described above will likely be subject to continuous change to improve robustness, sample throughput, and reduce costs, with the aim of providing life science laboratories with routine access to genomic information.

8.9.7 Microfabricated Devices

The 1990s saw the birth of a new paradigm for analytical instruments, conceived as fully integrated analytical systems fabricated on a single small-scale substrate using established concepts in electronic microchip technology. Conceptually, it is assumed that liquids can be manipulated in a network of microchannels in much the same way as electrons in microcircuits. These systems are popularly known as lab-on-a-chip, miniaturized total analysis systems (μ -TAS) or microfabricated devices [528-532]. Electromigration techniques appear prominently in this technology for systems containing a separation step [530-537]. Application of a voltage to the terminals of a microchannel is much easier to realize from an engineering perspective than pressure differences, because no moving parts such as pumps or valves are required. Off-chip pumps are not easily miniaturized, and operate at flow rates that are generally too high without splitting, for microfabricated devices. The ease of implementation of electromigration techniques compared with the comparative difficulty of packing columns on a microchip and designing a suitable pump are the main reasons for the limited number of systems utilizing chromatographic separation steps [528-530,536].

The potential advantages of microfabricated devices include: improved performance, measured in terms of separation speed and sample throughput; reduced manufacturing and operational costs due to the small footprint, and reduced resource consumption and waste production; and use of parallel processing for high sample throughput. Routine applications are expected to occur in the life sciences for genomics (DNA sequencing), proteomics (protein separations) and screening of combinatorial libraries [525,538,539]. For many of these applications, one-time-use only devices are desirable, with all processes integrated on the microchip, or sample processing performed on the microchip and reading of the results in a separate detection unit.

Electrophoresis microchips are mainly fabricated from various silica and glass substrates (but not silicon commonly used for semiconductor microchips). Vertical sidewalls are difficult to etch in these substrates and sealing glass plates usually require a cleanroom environment and high temperatures or voltages that are incompatible with most materials used in separation systems. Glass structures are fragile, and the multistep fabrication process is time consuming, increasing interest in polymer microchips, as a way of reducing manufacturing costs (albeit that most common polymers do not support a vigorous electroosmotic flow). The fabrication procedures for polymers are different from those for glass. Microfabricated devices on glass are usually prepared by standard photolithographic technologies [528,529,534]. Common techniques for polymer microchips include laser ablation, injection molding, silicone rubber casting and hot embossing. The design of microchips for capillary electrophoresis has undergone significant evolution from simple single-channel structures too increasingly complex ones. Recent designs incorporate precolumn and postcolumn reactions and separations in parallel channels with depths of 15-40 μm and widths of 60-200 μm . The small cross section of the separation channels and large thermal mass of the microchips allows efficient heat dissipation and the use of high field strengths (e.g. > 2 kV/cm). Short channel lengths are suitable for fast separations with band broadening (ideally) controlled only by diffusion. Longer channels require an increase in the length of the substrate, or can be created as a serpentine channel on a smaller substrate (e.g. 16.5-cm serpentine channel can be etched on a 1-cm² substrate). Sample zones migrating through a serpentine channel experience additional dispersion due to differences in the migration path at the inner and outer perimeter of each turn. The contribution to the plate height depends on the channel design and diffusion coefficients [540]. Total plate numbers depend on the applied voltage only, but the peak capacity and separation time depends on the channel length. Either reducing the channel length, or increasing the applied voltage, can increase the separation speed. Eventually, electrical breakdown of construction materials or air limits the applied voltage.

The simplest design for an electrophoresis microchip consists of microchannels laid out in cross geometries, with a straight separation channel intersected by a second channel for sample injection, Figure 8.15. Four fluid reservoirs are positioned at the channel ends, two for introduction of sample and electrolyte solution, the other two serving as waste reservoirs. The integrated injectors are usually either cross-channel injectors, formed by orthogonally intersecting the separation channel with

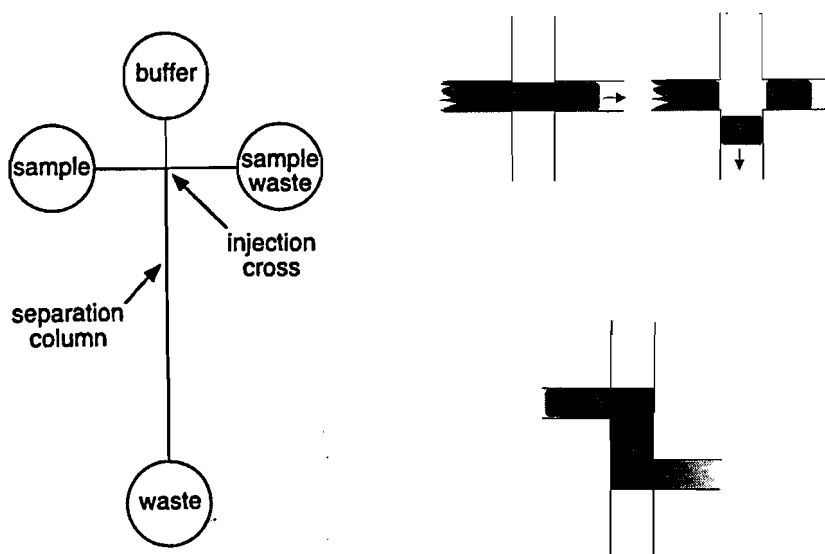


Figure 8.15. Diagram of a simple cross design for electrophoresis on a microchip (left) and illustration of cross injection and double-T injection systems (right).

a channel connecting the sample to waste, or twin-T injectors, where the two arms of the sample to waste channel are offset to form a larger injection region. A voltage applied between the sample reservoir and the opposite waste reservoir allows sample material to be pulled across the cross-junction. Switching off this voltage and applying a second voltage between the electrolyte solution reservoir and the opposite waste reservoir pulls sample into the separation channel. Integrated sample injectors permit volume-defined electrokinetic injection of short sample plugs of picoliter volumes with acceptable reproducibility ($RSD < 4\%$). Band broadening of the plugs can occur from leaks at the injection channel intersection. Electrical biasing of the different reservoirs, such as in pinched injection, minimizes sample bleed [541]. Sample stacking (section 8.9.3.1) can be used to enhance detection limits. Sample adsorption on channel walls and unstable electroosmotic flow are possible problems for glass microchips. Sensitive detection schemes are essential in microfabricated devices due to the extremely small size of the injected sample and detection volumes. Laser-induced fluorescence (LIF) with a confocal optical arrangement is the only common detection method at present. The large size of these detection devices reduces one of the advantages of microfabricated devices, namely the small dimensions of the rest of the experimental setup. Plasma sputtered platinum electrodes facilitate on-chip electrochemical detection. Both fluorescence and electrochemical detection are selective detection techniques, unsuitable for the detection of many sample types. On-chip derivatization reactions can be used to increase the scope of fluorescence detection, but at the expense of a more complicated microchip design. Absorption detectors can

be used, but detection sensitivity is a concern because of the limited optical path length of the separation channel for on-column detection.

8.10 REFERENCES

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9.1 INTRODUCTION

A chromatogram provides information about the complexity (number of components), quantity (peak height or area) and identity (retention parameter) of the components in a mixture. Of these parameters the certainty of identification based solely on retention is considered suspect, even for simple mixtures. When the identity can be firmly established the quantitative information from the chromatogram is authoritative. Spectroscopic techniques are plagued by the opposite problem. These techniques provide a rich source of qualitative information from which identity may be inferred with reasonable certainty. Spectroscopic techniques, however, are limited by two practical considerations: it is often difficult to extract quantitative information from their signals, and for reliable identification, pure samples are generally required. Since chromatographic and spectroscopic techniques provide complementary information, their combination provides a more powerful analytical tool than either technique alone. This provided the driving force for the development of combined or "hyphenated" systems of the type summarized in Table 9.1. Certainly other combinations have been described, but in this chapter the discussion is restricted to those techniques that are either widely used, or likely to become so, in the near future. Ideally, any combined

Table 9.1

Summary of common spectroscopic techniques used in a hyphenated form in chromatography

Chromatographic technique	Spectroscopic technique	Abbreviation
Gas Chromatography	Mass Spectrometry	GC-MS
	Infrared Spectrometry	GC-IR or GC-FTIR
Liquid Chromatography	Mass Spectrometry	LC-MS
	Infrared Spectrometry	LC-IR or LC-FTIR
	Nuclear Magnetic Resonance	LC-NMR
Supercritical Fluid Chromatography	Mass Spectrometry	SFC-MS
	Infrared Spectrometry	SFC-IR or SFC-FTIR
	Nuclear Magnetic Resonance	SFC-NMR
Capillary Electrophoresis	Mass Spectrometry	CE-MS
	Nuclear Magnetic Resonance	CE-NMR
Thin-Layer Chromatography	Mass Spectrometry	TLC-MS
	Infrared Spectrometry	TLC-IR or TLC-FTIR
	Surface Enhanced Raman Spectroscopy	TLC-SERS

instruments should operate under conditions that do not compromise the performance of either system alone, and do so in a continuous and automated manner organized by a system controller, which sets and monitors system components, and acquires and stores all aspects of the analytical data. To achieve this goal has presented various levels of difficulty for the techniques identified in Table 9.1. As well as general instrument aspects and their sympathetic relationship to separation conditions, most combined instruments require the construction of an interface to enable comprehensive automated operation, which in turn has been one of the main problems in the design of user friendly systems.

9.2 MASS SPECTROMETRY

A mass spectrometer produces a mass spectrum, which is a histogram of the relative abundance of the ions generated by ionization of the sample and their subsequent separation, based on their mass-to-charge ratio (m/z). The mass spectrum is a fingerprint of the molecule conveying information about its molecular weight, and if fragmentation occurs during ionization, or is brought about by collision induced dissociation, structurally useful fragment ions characteristic of the bond order of the structure. Through interpretation, the molecular substructures can be reassembled into the parent molecule using sets of rules and intuition, allowing the molecule to be identified, even if its mass spectrum was previously unknown, at least in favorable cases. Alternatively, computer matching to a library of known spectra can automatically identify an unknown mass spectrum, if the mass spectrum is present in the library. In addition, accurate mass measurements under high-resolution conditions allow an empirical formula to be assigned to the sample.

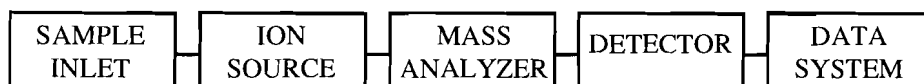


Figure 9.1. Block diagram of the main components of a mass spectrometer.

Table 9.2

Methods for producing ions in mass spectrometry with a chromatographic sample inlet

MM = molecular mass

Ionization method	Typical ions	Sample types
Electron Impact (EI)	$M^{+\bullet}$, fragment ions	Non-polar and some polar organic compounds with MM < 1200 Da
Chemical Ionization (CI)	MH^+ , $(M-H)^+$, $(M+RH)^+$ M^- , $(M-H)^-$	Non-polar and some polar organic compounds with MM < 1200 Da
Atmospheric Pressure	MH^+	Polar and some non-polar organic compounds with MM < 1000 Da
Chemical Ionization (APCI)	$(M-H)^-$	Polar organic compounds and polymers with MM < 200,000 Da
Electrospray (ESI)	MH^+ , $(M+nH)^{n+}$ $(M-H)^-$, $(M-nH)^{n-}$	Polar organic compounds and polymers with MM < 200,000 Da
Thermospray (TSP)	MH^+ , MNH_4^+ $(M-H)^-$	Polar and some non-polar organic compounds with MM < 1000 Da
Fast Atom Bombardment (FAB)	MH^+ $(M-H)^-$	Polar organic compounds, salts and polymers with MM < 10,000 Da
Matrix-Assisted Laser Desorption/Ionization (MALDI)	MH^+ $(M-H)^-$	Polar and some non-polar polymers with MM < 200,000 Da.

A block diagram of the main components of a mass spectrometer is presented in Figure 9.1. Individual stable compounds, and possibly simple mixtures, are conventionally introduced into the ion source by a heated direct inlet probe for liquids and solids, or by a vacuum leak for volatile liquids and gases [1-7]. Thermally labile and ionic compounds, and some polar compounds, in solution are introduced by a continuous infusion mechanism, and ionized by one of the soft ionization techniques, such as electrospray. For mixtures, in general, a chromatographic sample inlet is the preferred choice.

The ion source is responsible for creation of an ion beam containing ions representative of the sample, and in a form suitable for separation by the mass analyzer. A number of different methods are used for ionization in conjunction with chromatographic sample inlets, Table 9.2, and are described shortly (section 9.2.1) [1-8]. In selecting the ionization method the molecular mass, volatility, thermal stability, polarity, and ion stability are all-important factors. There is no universal ionization method, and all methods listed in Table 9.2 have specific applications, but the general use of thermospray and fast atom bombardment is in decline, being superseded to a large extent by atmospheric pressure ionization methods. The purpose of the mass analyzer is to separate the ions formed in the ion source by differences in their mass-to-charge ratio. Dispersion in a magnetic field (magnetic sector), mass selection in an oscillating electric field (quadrupole or ion trap), velocity differences in a drift tube

(time-of-flight) or oscillations in a magnetic field (Fourier transform-ion cyclotron resonance) are used to achieve this. Fourier transform ion cyclotron resonance mass spectrometry, or simply Fourier transform mass spectrometry (FTMS), is a versatile and high resolution technique that is limited for chromatographic applications primarily by cost and complexity more so than performance, and will not be discussed here [1,9,10]. The performance of a mass analyzer can be expressed in terms of its operational mass range, mass resolution, ion transmission properties, scan speed, and ease of coupling with other mass analyzers in hybrid instruments. For typical chromatographic applications a modest mass range and mass resolution is often acceptable to obtain a high ion transmission (sensitivity). In addition, a high scan speed is important to minimize variation of the ion abundance across a chromatographic peak. For ion detection, an electron multiplier is commonly used, or less frequently a Faraday cage collector. Multichannel array detectors are sometimes used for simultaneous detection of ions with different mass-to-charge ratios using magnetic sector instruments.

The general description mass spectrometer covers a variety of instruments that vary in size, cost, versatility, performance and operational complexity, but all find specific applications appropriate to their design. The common use of mass spectrometers as chromatographic detectors has resulted in a significant market for low cost instruments with modest performance characteristics that are easy to operate, robust, and require little bench space. Instruments in this category will be the focus of the following sections.

9.2.1 Ion Sources

Organic compounds can be ionized in many ways; those of primary interest for use with chromatographic inlets include electron impact, chemical ionization, atmospheric pressure ionization (electrospray and atmospheric pressure chemical ionization), thermospray, and fast atom bombardment [7,8].

9.2.1.1 Electron Impact

Electron impact is used to ionize neutral and volatile compounds in a heated, evacuated chamber by energy exchange during collisions with an electron beam of narrow energy distribution. Electrons are generated from a heated metal filament outside the ion source box, and accelerated by a potential difference between the filament and the ion source box, Figure 9.2. The electrons then pass through small entrance and exit apertures in the box, and are collected by a trap electrode. Sample vapors enter the heated ionization box, where they interact with the electron beam at pressures below about 10^{-5} Torr. Low pressures are required to ensure that the average mean free path of the ions formed in the source is sufficient for them to escape the source without alteration by ion-molecule collisions. A voltage applied to a repeller electrode within the source box directs and accelerates the ions towards the ion exit aperture. A collimated magnetic field, applied parallel to the electron beam axis, allows a high transmission of the electrons with minimal perturbation of the ion beam. The electron current is stabilized by monitoring

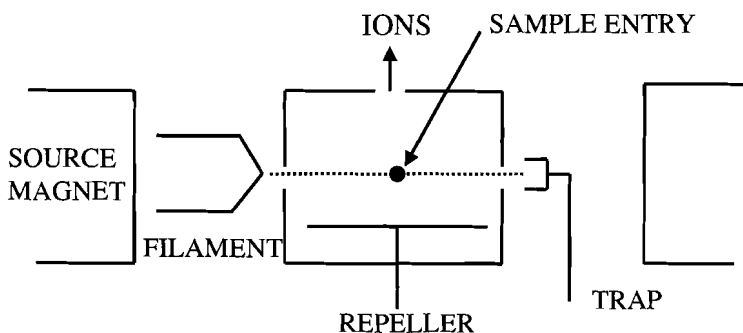


Figure 9.2. Schematic diagram of a typical electron impact ion source. (From ref. [8]; ©American Chemical Society).

either the total emission current, or trap current, with feedback to the filament heater power supply to maintain a constant current.

The electrons have energies selectable between 5 to 100 eV, with value of 70 eV adopted as a standard value for recording reference mass spectra. Since most organic compounds have ionization potentials of 7 to 20 eV, the energy transferred on collision between the electron and a neutral molecule is sufficient to cause both ionization and extensive fragmentation. The majority of ions formed by this process are singly charged odd-electron molecular and fragment ions together with a few multiply charged ions and some negative ions. Except for compounds of high electron affinity, the number of stable negative ions formed is only a small fraction of the total number of ions formed, and for all practical purposes, electron impact is essentially a positive ion source.

There are two limitations of electron impact ionization, which in other ways is nearly an ideal ion source for identification of organic compounds. The sample must be stable as neutral molecules in the gas phase prior to ionization. There are many compounds, however, that are simply not stable at the temperatures required for vaporization. Radical molecular ions are formed with excess energy that may be sufficient for complete elimination of molecular mass information from the mass spectrum, even at low electron energies (e.g. 15-20 eV). Molecular mass information is almost always required for structure elucidation of unknown compounds. A softer ionization technique, such as chemical ionization, may be used in these circumstances to establish the compound molecular mass.

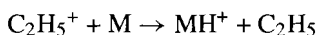
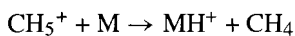
9.2.1.2 Chemical Ionization

Chemical ionization mass spectra are produced by ion-molecule reactions between neutral sample molecules and a high-pressure (0.2-2 Torr) reagent gas ion plasma in a source otherwise similar to an electron impact source [4,7,8,11]. A reagent gas is introduced separately and continuously at a higher concentration than the sample into the ion source box. The reagent gas plasma is generated by electron impact using higher energy electron beams (200-1000 eV) than for electron impact sources, to ensure

sufficient penetration of the ionizing electrons into the active source volume. The source region is described as "tight", indicating the use of smaller orifices and slits to allow efficient operation at higher pressures than the electron impact source. Differential pumping or a larger vacuum pump is commonly used to accommodate the greater pressure difference between the ion source and the mass analyzer regions of the mass spectrometer.

The essential requirement for the reaction gas is that it affords a set of ions, which do not react with themselves, but can undergo energetic ion-molecule reactions with the sample. The electron beam primarily ionizes the reagent gas owing to its higher concentration. Collisions between the sample and reagent gas plasma occur at thermal energies, such that the energy available to drive fragmentation is limited to the reaction energy. Furthermore, ionization occurs at relatively high pressures, where collisional relaxation can compete with fragmentation. Thus, these collision processes are gentler than electron impact ionization, and produce stable molecular ions or molecular ion adducts with little additional fragmentation. The high-pressure conditions in the chemical ionization source also favor the production of thermal electrons, which may be captured by molecules with a high electron affinity, to form negatively charged molecular ions, or molecular ion fragments, after dissociation [11-13]. The negative ion concentration may be two or three orders of magnitude greater than for electron impact conditions, making negative ion chemical ionization mass spectrometry a viable method. Its areas of application parallel those of the electron-capture detector for gas chromatography (section 3.10.1.4), to which a number of mechanistic similarities exist. Stable adduct complexes may be formed by nucleophilic addition with anions of low proton affinity, such as Cl^- and $\text{O}_2^{\bullet -}$, which cannot usually be ionized by proton transfer. Many modern instruments support the detection of either positive or negative ions with nearly equal facility, but this is not always the case [1-4].

The reagent gas plasma can ionize neutral molecules by collision induced processes involving proton transfer, anion abstraction, charge exchange, and electrophilic addition (adduct formation). Most reagent ions are capable of participating in more than one type of reaction. For methane, one of the most widely used reagent gases, at a source pressure of approximately one Torr the dominant plasma ions consist mainly of CH_5^+ (48%), C_2H_5^+ (40%) and C_3H_5^+ (6%). In the gas phase, CH_5^+ and C_2H_5^+ ions function as strong acids and can transfer a proton to the sample, as shown below.



The C_2H_5^+ ion can also function as a Lewis acid, forming collision-stabilized complexes or molecular ion adducts:

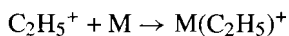


Table 9.3

Prominent reagent ions formed under chemical ionization conditions

Reagent gas	Prominent reagent ions at a pressure ≈ 1 Torr
Methane	CH_5^+ , C_2H_5^+ , C_3H_5^+
Propane	C_3H_7^+ , C_3H_8^+
Isobutane	C_4H_9^+
Hydrogen	H_3^+
Ammonia	NH_4^+ , $(\text{NH}_3)_2\text{H}^+$, $(\text{NH}_3)_3\text{H}^+$
Water	H_3O^+
Tetramethylsilane	$(\text{CH}_3)_3\text{Si}^+$
Dimethylamine	$(\text{CH}_3)_2\text{NH}_2^+$, $[(\text{CH}_3)_2\text{NH}]_2\text{H}^+$, $\text{C}_3\text{H}_8\text{N}^+$
Helium	He^+

The product ions generated by chemical ionization are stable, even-electron species, with relatively little excess energy compared to those generated by electron impact. The chemical ionization mass spectrum, therefore, is characterized by the presence of a few intense molecular ion adducts with very little further fragmentation from which the sample molecular mass is readily identified from the m/z value of the molecular ion adducts. The type of molecular ion adduct formed depends mainly on the sample composition and the identity of the reagent gas.

Some typical reagent gases, and the predominant ions formed in the reagent gas plasma, are summarized in Table 9.3. The most common reaction pathway for these reagent ions is to protonate the sample and produce dominant MH^+ ions, so long as the proton affinity of the sample exceeds the proton affinity of the reagent ion. Methane protonates most compounds, while reactions with isobutane are more selective and considerably less exothermic. Fragmentation, often observed with methane, may be largely absent in the isobutane chemical ionization mass spectrum. Solvents such as water, methanol, and ammonia are also relatively mild protonating reagents. With tetramethylsilane, trimethylsilylation rather than protonation is observed. Dimethylamine selectively ionizes carbonyl-containing compounds, with protonated dimethylamine apparently attached to the carbonyl group. With aldehydes and esters, hydride abstraction occurs. Ammonia is also an electrophilic addition reagent, forming abundant $(\text{M} + \text{NH}_4)^+$ ions with a variety of compounds. The noble gases He, Ar, and Xe cause ionization by a charge exchange forming predominantly odd electron molecular ions. The appearance and reproducibility of chemical ionization mass spectra depends on the ionizing conditions, principally the source temperature and pressure, and the purity of the reagent gas. Chemical ionization mass spectra are generally not as reproducible as electron impact mass spectra, and libraries of chemical ionization mass spectra are not available for compound identification by library search methods.

Chemical ionization with an ion trap mass spectrometer is possible at much lower-reagent gas pressures and longer reaction times than typical external sources. A preliminary ionization period is used to establish the reagent gas ion concentration, followed by a relatively long (10s of ms) reaction period at a slightly higher radio frequency potential, during which sample ions are formed and stored. The radio

frequency potential is then ramped to affect the mass scan in the usual way. The chemical ionization mass spectra obtained in this way are sometimes different to conventional chemical ionization mass spectra obtained from scanning instruments.

9.2.1.3 Atmospheric Pressure Chemical Ionization

Atmospheric pressure ionization techniques, which include atmospheric pressure chemical ionization (APCI) and electrospray ionization (section 9.2.1.4), are the most widely used approach for LC-MS applications. Atmospheric pressure chemical ionization was earlier used for GC-MS, but has almost been abandoned for this purpose, except for some specific applications (e.g. gas purity analysis) [14]. The APCI source/interface (section 9.2.2.2) consists of a heated pneumatic nebulizer that converts the liquid flow into a spray of fine droplets. The droplets migrate with the support gas through a desolvation chamber to the ion formation region, where a corona discharge initiates chemical ionization at atmospheric pressure using the vaporized solvent as the reagent gas [1,4,7,8,11,14,15]. The corona discharge needle is held at a positive potential of 2-3 kV for positive ions (negative for negative ions), and is responsible for ionizing the solvent molecules. A combination of collisions and charge exchange reactions between solvent and gas molecules forms a reagent gas plasma, localized in the region of the corona discharge needle. This reagent gas plasma is responsible for ionizing sample molecules by proton transfer or abstraction to produce MH^+ or $(M-H)^-$ ions mainly with possibly some adduct ion formation. Compounds of high electron affinity may form negative ions by electron capture.

The composition of the reagent gas plasma is controlled by the mobile phase composition. It contains reactant ions of the type H_3O^+ , $CH_3OH_2^+$, CH_3CNH^+ , etc., in most cases solvated by solvent molecules, for example, $CH_3OH_2^+(H_2O)_n(CH_3OH)_m$ with $n + m \leq 4$. The presence of ammonia vapor, for example, as a result of the evaporation of ammonium acetate added to the mobile phase, causes rapid removal of protonated solvent ions with formation of ammonium ions that become the dominant reagent gas ions. The addition of alkylamines or acids to the mobile phase results in the formation of protonated amines or anions as the dominant species in the reagent gas plasma. Since highly reactive ions, such as CH_5^+ , can not be formed by APCI in the presence of solvents, it is not possible to ionize saturated hydrocarbons or other compounds of low gas-phase basicity.

Atmospheric pressure chemical ionization results in the formation of mainly molecular adduct ions suitable for indicating the molecular mass, but the absence of significant fragmentation, limits possibilities for structure elucidation. Fragmentation can be induced by collision induced dissociation (CID) using either tandem mass spectrometry (section 9.2.4) or by reactions in the transport region of the interface/source. In the transport region the gas pressure is still relatively high, and creates the necessary conditions for CID and efficient collection of the fragment ions. In source CID is usually induced by application of an appropriate voltage difference between two regions of the ion source, for example, between the nozzle cone and the skimmer, or between the skimmer and the ion guide, depending on the source design. The same approach

is employed for in source CID using electrospray ionization, and a general discussion of the effect of experimental parameters on the quality and reproducibility of the mass spectra, is deferred to the next section.

9.2.1.4 Electrospray

Electrospray is viewed as the most versatile ionization technique for neutral compounds and ions in solution, and at the same time a general-purpose interface for LC-MS [14-24]. In electrospray ions are formed in solution and then transferred to the gas phase. This differs from APCI where neutral molecules are first transferred to the gas phase and then ionized by gas-phase ion-molecule reactions. The heart of the electrospray source is a metal capillary through which the sample solution flows. A potential of 3-6 kV is applied to the capillary forming a spray of fine droplets directed towards a counter electrode with a sampling orifice located about 1-3 cm from the capillary tip. A positive potential is applied to the capillary to generate positive ions, and a negative potential for negative ions. To accommodate different liquid flow rates droplet formation is assisted by optimization of the orifice diameter of the capillary sprayer, the use of a coaxial gas flow, and heat to increase the rate of solvent evaporation.

There are three major steps in the production of gas phase ions by electrospray: the formation of charged droplets at the capillary tip; the shrinkage of the charged droplets by solvent evaporation and repeated droplet disintegrations; and production of gas phase ions from the very small and highly charged droplets. The production of positively charged droplets, for example, results from the removal of negative charge via electrochemical discharge of negative ions at the metal wall of the spray capillary. The accumulated charge at the liquid surface leads to the formation of a liquid cone at the capillary tip that breaks up into a fine jet with the production of charged droplets. These droplets are small (about 1 μm) with a narrow size distribution. They undergo size reduction by solvent evaporation as they move towards the sampling orifice. At the point where the Coulomb repulsion forces between charges exceed the surface tension of the drop (Rayleigh instability limit), the resulting instability tears the droplets apart, producing smaller charged droplets. These droplets lose further solvent molecules by evaporation and disintegrate again to smaller droplets. According to the ion evaporation model, when a droplet size of about 10 nm is reached, the charge density becomes so high that ions are desorbed from the droplet into the gas phase. Alternatively, according to the charge residue model, the succession of droplet size reductions results in the formation of fine particles that contain at most one ion. Solvent evaporation from these fine particles leads to ion transfer to the gas phase.

For compounds less than 1000 Da, electrospray produces mainly protonated molecular ions MH^+ for basic compounds and deprotonated molecular ions $(\text{M-H})^-$ for acidic compounds. Some adduct ions containing ammonium $(\text{MNH}_4)^+$, sodium $(\text{MNa})^+$ or $(\text{M-H+Na})^-$, potassium $(\text{MK})^+$ or formate $(\text{M-H+HCO}_2)^-$ may be observed as well, and sometimes can be exploited to detect compounds inefficiently ionized by proton transfer [25]. Higher molecular mass compounds produce multiply charged ions of the type $(\text{M+nH})^+$ or $(\text{M-nH})^-$ under electrospray conditions, for example

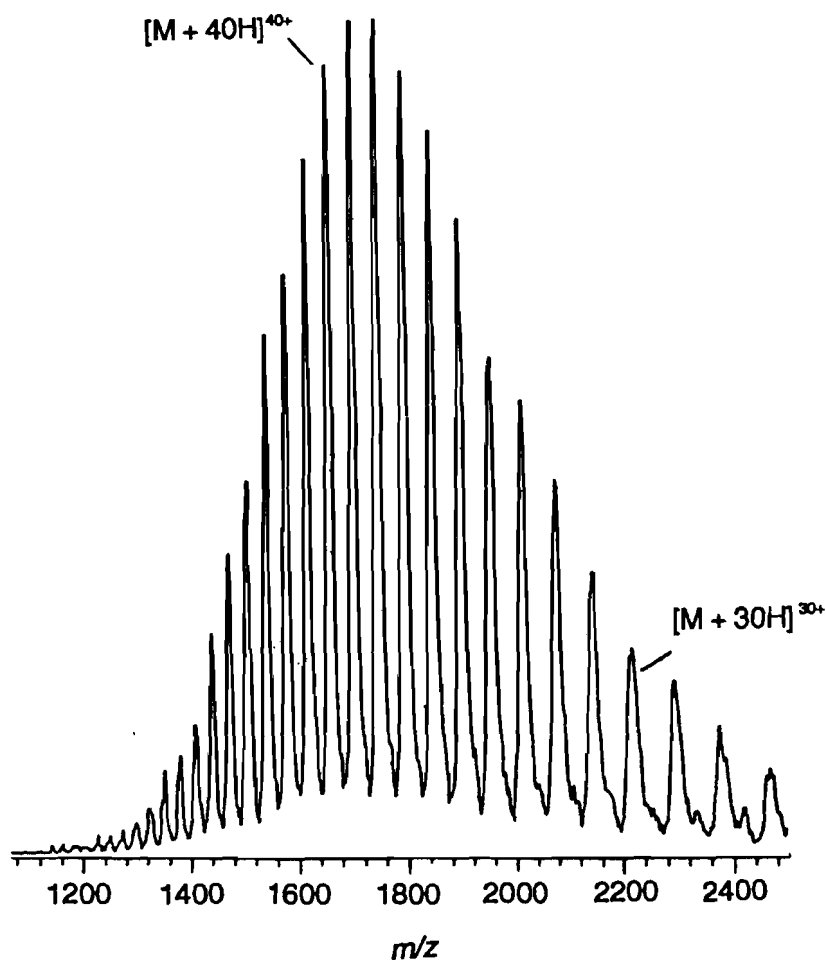


Figure 9.3. Positive-ion electrospray mass spectrum of bovine serum albumin of molecular mass 66,300. (From ref. [21]; ©Bentham Science Publishers).

Figure 9.3, where n is the number of charges on the ion. This mechanism is exclusive to electrospray, and allows mass analyzers with a modest mass range to be used to identify the molecular mass of compounds well beyond their normal scan range. Since the mass spectrometer separates ions according to their m/z value multiply charged ions of mass $> 100,000$ tend to appear in the range m/z 300 to 4000, easily accessible to quadrupole and similar mass-range limited mass analyzers. Multiple charging in electrospray creates a whole series of molecular adduct ions each of which represents a different charge state for the same compound. The charge state can be determined if it is assumed that adjacent peaks in the series differ by only one charge. For example, for positive

ions $n = (M_2 - H) / (M_1 - M_2)$ where M_1 and M_2 are the m/z values for two consecutive molecular adduct ions in the electrospray mass spectrum. Once the charge state has been determined, the molecular mass can be estimated for each ion and the values averaged to provide an accurate value for the molecular mass. Automated calculation of molecular mass by software is a generally supported operation. Electrospray and MALDI are credited with establishing mass spectrometry as an important tool for the sequencing and identification of high molecular mass synthetic polymers and biopolymers.

Electrospray mass spectra are generally devoid of fragment ions required for structure elucidation when identification from molecular mass is insufficient. Fragmentation can be achieved in different ways, either by varying electrospray source parameters to induce in-source fragmentation, or more specifically, by using tandem mass spectrometry (section 9.4.2). By increasing the sampling cone or ion guide voltage relative to the skimmer voltage, ions enter the transport region of the interface with sufficient energy for collisions with solvent and gas molecules to result in fragmentation. Optimization and standardization of this method are difficult, however, due to differences in source and interface designs [26-30]. These difficulties may be circumvented to an extent by use of breakdown curves for tune compounds to establish somewhat similar operating conditions to generate searchable mass spectral libraries. In general, more energy is required to induce fragmentation of larger ionized molecules as internal energy obtained from collisions can be partitioned over a larger number of vibrational degrees of freedom. This makes the choice of an optimum fragmentation voltage for acquisition of library spectra and for recording spectra of unknown compounds more difficult. At present there are no commercial electrospray (or APCI) spectral libraries available for structure identification by LC-MS. Several research groups have developed modest mass spectral libraries for drugs, pesticides and explosives by in-source collision induced dissociation [29,30]. Two different concepts for setting-up in-source collision induced dissociation libraries for compound identification by computer matching of unknown mass spectra have been developed. Composite mass spectra obtained by averaging two spectra, one at low and one at high fragmentation voltages, or three single mass spectra with different collision energies (e.g. 20, 50, 70 eV) for each compound are entered in the library. For practical applications, library search parameters are set in a way that only minor weight is given to the relative ion abundance. Structure elucidation and identification of unknown compounds by searching compound libraries of reference spectra is by no means as reliable, or robust, as methods developed for electron impact mass spectra (section 9.2.5.3).

9.2.1.5 Thermospray

Thermospray was in several ways the forerunner of electrospray and atmospheric pressure chemical ionization sources, and in current practice has largely been superseded by them. Thermospray (TSP) is a combination of interface and ion source for LC-MS and other liquid-phase sample introduction systems [7,31-33]. A continuous flow of liquid is rapidly vaporized in a resistively heated metal capillary tube forming a supersonic

jet of small droplets, at least some of which are electrically charged, when the solution is partly aqueous and a volatile buffer, such as ammonium acetate, is also present. An equal number of positively and negatively charged droplets are formed leading to the production of both positive and negative sample ions. Most thermospray sources are fitted with a filament or discharge electrode to assist in ion production. Electrons from the filament (filament-on) or discharge (plasmaspray) ionizes the solvent vapor, and hence the sample, by a chemical ionization processes.

The presence of an electrolyte (e.g. 50-100 mM ammonium acetate) is particularly important, since without it the ion yields are low. The initial droplets created by the spray continue to shrink due to evaporation of solvent induced by heat and vacuum as they travel through the source towards the orifice at the entrance of the mass analyzer. Eventually they become sufficiently small for free ions to be ejected from the droplets. Alternatively, ions can be generated in a two-step process similar to conventional chemical ionization, whereby an ion of the electrolyte, e.g. NH_4^+ , is ejected from the droplet, reacts with a sample molecule in the gas phase, and generates a sample ion. The ion types observed by either process are usually molecular adduct ions, such as MH^+ , $(\text{M}+\text{NH}_4)^+$, $(\text{M}-\text{H})^-$, $(\text{MO}_2\text{CCH}_3)^-$, etc., accompanied by a few low intensity fragment ions. Solvent sample complexes are also possible, e.g. $(\text{M}+\text{CH}_3\text{CN})\text{H}^+$ and $(\text{M}+\text{CH}_3\text{CN})\text{NH}_4^+$, and can complicate the spectrum. Thermospray is by no means a universal ionization process, and in some cases, it may be necessary to boost ion production using filament-on or plasma discharge ionization. For samples in low polarity solvents that do not dissolve volatile electrolytes, this becomes obligatory.

9.2.1.6 Fast Atom Bombardment

For fast atom bombardment (FAB), the sample is dissolved in a viscous liquid (matrix) and spread as a thin film on a metal target [1,7,34-36]. Bombardment of the solution by a beam of high energy particles results in the expulsion of material into the gas phase by momentum transfer (sputtering), in which some of the sputtered material will be in the form of both positively and negatively charged ions. These ions are extracted by a slit lens system and directed to the mass analyzer. The particle beam is usually comprised of inert gas atoms with energy between 3-8 keV or cesium ions of higher energy (< 35 keV). In the latter case, the technique is also known as liquid secondary ion mass spectrometry (LSIMS). Such a distinction, however, is unnecessary for a discussion of chromatographic applications, where the source is operated in the continuous flow mode as an interface and ion source (section 9.2.2.2).

The mass spectrum is dominated by the formation of even electron molecular ion adducts of the type MH^+ and $(\text{M}-\text{H})^-$, sometimes accompanied by cluster ions containing the molecular ion adducts together with different numbers of neutral parent or matrix molecules, e.g. $(\text{M}_n+\text{H})^+$, $(\text{M}-\text{H}+\text{M}_n)^-$ and $(\text{MH}+[\text{Matrix}]_n)^+$ with $n = 1-5$. In addition ions of the type $(\text{MNa})^+$ or $(\text{MK})^+$ from sample impurities or intentional doping may be observed as well. In most cases, sputtering and ionization occur with the formation of a small number of fragment ions. As well as sample-related ions, abundant background ions attributed to protonated matrix clusters of the type $([\text{Matrix}]_n+\text{H})^+$

with $n = 1-10$, are observed. In general, the low-mass region of the mass spectrum contains a high chemical background originating from the matrix, and it is difficult to detect ions with $m/z < 200$. FAB is widely used for the analysis of thermally labile and polar compounds, and ionic compounds with molecular mass < 4 kD. Higher molecular mass compounds can be analyzed by FAB, but MALDI and electrospray are generally preferred for these samples.

The exact mechanism of the ionization process is unknown, but at least three possibilities seem to be important. Desorption of preformed ions by energy transfer upon particle impact. Desolvation of preformed ions in the droplets ejected from the liquid surface upon particle impact. Gas phase ion-molecule interactions near the liquid surface-vacuum interface. The matrix composition plays a dominant role in the ion production process. It controls the solution chemistry leading to preformed ions, the renewal of the surface layer from which preformed ions are desorbed, reduces the energy required for desorption by ion solvation, and provides the reagent for gas phase chemical ionization. Suitable matrix solvents are viscous liquids with a low vapor pressure, have good solubilizing properties, and suitable protonation properties and surface activity. Typical examples include glycerol, thioglycerol, tetraglyme, ethanolamine, and diethanolamine.

9.2.1.7 Matrix-Assisted Laser Desorption/Ionization

Matrix-assisted laser desorption/ionization (MALDI) is widely used for the formation of gas phase molecular ion adducts from large biomolecules (e.g. proteins, oligonucleotides and oligosaccharides) and high molecular mass synthetic polymers [5,36-41]. Ionization occurs concurrently with laser ablation from a solid solution of the sample in a light-absorbing matrix. The sample solution is mixed with a large molar mass of matrix and a few μl or less deposited as a droplet on a multiwell metal target. The solvent is evaporated and the target placed in the evacuated MALDI source and each sample bombarded successively with the laser beam. The most frequently used wavelength is 337 nm from a nanosecond pulsed nitrogen laser. The gas-phase ions are accelerated by an electric field towards the mass analyzer, in most cases a time-of-flight mass spectrometer. Time-of-flight is well suited to this application because of its nearly unlimited mass range and compatibility with pulsed-ion sources.

The function of the matrix is to transfer energy from the laser beam to the sample, to prevent aggregation of sample molecules, and probably to participate in the ionization process in some unspecified way. A large number of matrix compounds have been described, but selection criteria are not well developed. Typical examples include derivatives of benzoic and cinnamic acids, nicotinic acid, succinic acid, and anilines.

Owing to the relatively mild ionization process, the dominant ions formed include protonated molecular ions, molecular ion adducts, and cluster ions similar to those described for FAB (section 9.2.1.6). A number of different chemical and physical mechanisms have been proposed to explain ion formation, including gas-phase photoionization, ion-molecule reactions, disproportionation, excited state proton transfer, energy pooling, thermal ionization, and desorption of preformed ions [37]. The choices of ma-

trix and laser wavelength are crucial parameters determining the ionization mechanism, but other factors are also important. Because the critical step in the sample preparation technique involves crystallization, the compatibility between MALDI and chromatographic processes is poor, and direct coupling is difficult to achieve. On-line coupling of capillary electrophoresis and liquid chromatography to MALDI ion sources have been described using a continuous-flow probe, similar to FAB (section 9.2.1.6), or aerosol spray with gas phase laser ionization [39-41]. The simplest and most widely used approach, however, is fraction collection followed by conventional sample preparation.

9.2.2 Chromatographic Interfaces

The function of an interface is to allow the chromatographic system and mass spectrometer to function at near optimum conditions by solving incompatibility problems between the two systems. Problems can arise because of the difference in material flow requirements, pressure gradients, presence of nonvolatile materials essential for the separation, and the desire to generate sample information without interference from the mobile phase in which it is diluted. In addition, the interface should not degrade the separation because of its volume, nor modify the sample composition because of thermal or chemical effects. For gas chromatography the primary incompatibilities are related to material flow and pressure gradients. The column outlet in gas chromatography is typically at atmospheric pressure while ion source pressures in the mass spectrometer are in the range of 2 to 10^{-5} Torr for chemical and electron impact ionization, respectively. The interface must be capable of providing an adequate pressure drop between the two instruments, maximize sample throughput, and maintain a gas flow rate compatible with the source operating pressure. Differentially pumped mass spectrometers can accept gas flow rates of about 1-2 ml/min (adjusted to STP) into the ion source. This is compatible with optimum flow rates for some open tubular columns, but is less than typical flow rates for wide-bore open tubular columns and packed columns. The gas burden is a bigger problem for liquid and supercritical fluid chromatography. A liquid chromatograph operating at a flow rate of 1.0 ml/min, for example, would produce nearly 200-1000 ml/min of vapor at STP. This is well in excess of the mass spectrometer acceptance rate, and requires a different solution to gas chromatography.

9.2.2.1 Gas Chromatography-Mass Spectrometry

Several interface designs are available for gas chromatography-mass spectrometry (GC-MS) [1,4,41-48]. For open tubular columns up to 0.32-mm internal diameter, either direct coupling or split-flow interfaces, are generally used. These columns operate at optimum flow rates less than 2 ml/min, which is entirely compatible with mass spectrometer vacuum systems. Flow splitting allows higher flow rates with some loss of sample. For wide-bore open tubular columns and conventional packed columns with flow rates higher than 5 ml/min an enrichment device, such as a jet separator, is generally preferred. Effusion separators (Watson-Biemann separator) and membrane separators are rarely used today.

Flexible, fused-silica open tubular columns are easily connected to the ion source with a simple vacuum-tight flange coupling and heated column support. This allows the position of the column end within the ion source to be adjusted for maximum ion yield. The sample is quantitatively transferred to the ion source, but the separation performance of the column may be compromised to some extent by the large pressure drop placed across it. In addition, the retention time of components will vary with changes in the source pressure. Another direct coupling device employs a fixed inlet restrictor to the mass spectrometer and a low-dead volume needle valve that acts as a flow diverter. This interface is more versatile, as it allows a wider selection of column flow rates, permits columns to be changed without instrument shutdown, and provides a by-pass by which large solvent volumes or corrosive derivatizing reagents can be vented away from the mass spectrometer. Disadvantages are the introduction of dead volume and the possibility of transforming labile compounds on the hot metal surfaces of the valve.

In several respects the use of an open split coupling is preferred over the valve interface. The open split interface essentially consists of a low-dead volume tube, swept by a stream of gas into which the column end or transfer capillary and a capillary restrictor tube from the mass spectrometer are inserted from either end, leaving a small gap between them. In some cases the restrictor capillary for the mass spectrometer is made narrower than the internal diameter of the column so that it can be inserted directly into the column end without completely sealing the column. The amount of sample entering the mass spectrometer depends on the ratio of the inlet flow rate of the ion source (which is fixed for a given inlet capillary, source conductance, temperature and carrier gas) to the column flow rate. The total sample is transferred to the mass spectrometer only if the outlet flow rate of the column equals the inlet flow rate of the ion source; hence, no split occurs. If the flow rate of the column is larger than that of the source inlet, part of the sample is split off at the interface and does not reach the mass spectrometer. If the column flow rate is less than the inlet flow rate of the source, the sample is diluted by makeup gas and the mass flow of sample to the ion source is reduced. Activating a valve in the makeup gas line provides a means of diverting solvent or reagent away from the mass spectrometer and then rapidly restoring the analytical conditions after a preset time. Other advantages of the open split interface are: the column end is always at atmospheric pressure, so that retention times are reproducible; the interface is versatile with respect to the use of different column diameters and flow rates; and the interface permits easy change of the separation column without affecting the operation of the mass spectrometer. It should be noted that the yield for the interface will remain constant throughout a temperature programmed separation only if the carrier gas is mass controlled or if the interface is heated at the same rate as the column (i.e. the interface is installed in the gas chromatographic oven).

The most popular interface for high flow rate columns is the jet separator, Figure 9.4. The effluent from the gas chromatograph is throttled through a narrow orifice where it rapidly expands into a heated vacuum chamber. During the expansion, the lighter carrier gas molecules rapidly diffuse away from the core of the supersonic jet, which

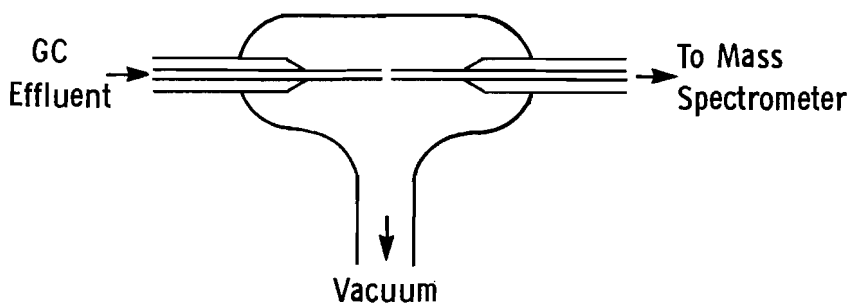


Figure 9.4. A jet separator for GC-MS with high-flow rate columns.

becomes enriched in the heavier sample molecules. The core is received by a second jet and enters the mass spectrometer. The alignment, orifice diameter, and relative spacing of the expansion and collection jets are crucial. Normally these are fixed at the time of manufacture so that maximum enrichment will only be obtained over a restricted flow range, sometimes requiring the use of makeup flow with some low flow rate columns. A dump valve is often introduced between the column and separator to divert solvent, etc., away from the ion source. The main deficiencies of the jet separator are that enrichment declines for samples of high volatility and plugging of the capillary restrictor by particles can render the interface inoperable.

9.2.2.2 Liquid Chromatography-Mass Spectrometry

The problems encountered in coupling liquid chromatography to mass spectrometry (LC-MS) were both more severe and different to those for GC-MS. It was not until the middle of the 1990s that LC-MS was able to penetrate the general analytical laboratory market, which GC-MS had dominated for over a decade by then. During this time interface designs for LC-MS were developed, modified, and finally rendered obsolete if they provided a poor compromise in terms of performance, complexity and robustness. Some of these early developments, reviewed elsewhere [31,41,49-52], have survived in an improved form in contemporary interface designs. Compared with the recent past, the current picture is far simpler. The majority of current LC-MS applications employ atmospheric pressure ionization sources based on electrospray or chemical ionization. Thermospray is still in use, but is being phased out in favor of atmospheric pressure ionization methods. Continuous-flow fast atom bombardment and particle beam interfaces continue in use for a few applications better handled by these techniques than atmospheric pressure ionization methods. Atmospheric pressure ionization methods produce predominantly molecular ion adducts with little fragmentation, but this disadvantage has become less important with the development of collision induced dissociation and tandem mass spectrometry techniques. These methods are capable of producing secondary fragmentation from selected primary ions, useful for identification purposes.

A typical atmospheric pressure ionization source consists of a capillary nebulizer for liquid introduction; an atmospheric pressure source region where ions are produced; ion sampling apertures and a vacuum system to reduce the pressure in the source region to a value compatible with the high vacuum of the mass analyzer; and an ion optical system to transport ions into the mass analyzer [14,17-19,23,41,53-59]. Initially atmospheric pressure ionization sources were developed for either electrospray or atmospheric pressure chemical ionization operation, but most commercial sources now support interchangeable use. Switching from electrospray to atmospheric pressure chemical ionization, and *vice versa*, only requires changing the liquid introduction probe and adding a corona discharge electrode for atmospheric pressure chemical ionization.

The column eluent is introduced into the atmospheric pressure ionization source as a fine spray originating from a narrow bore capillary aided by application of heat, a coaxial nebulizer gas stream, an electrostatic potential, or ultrasonic vibration of the capillary, or a combination of these. In electrospray droplet formation and charging take place simultaneously, while in atmospheric pressure chemical ionization droplets are formed first prior to subsequent gas-phase ionization. An electrospray is obtained by application of an electric field to a slow flow of a conducting liquid from a capillary tube. The inner and outer diameters of the spray tip and its position relative to the sampling orifice are optimized to improve ionization efficiency and ion sampling at different column flow rates. Coupling different column flow rates to electrospray is achieved by using either post-column splitting or optimizing the dimensions and position of the capillary sprayer. Capillary diameters of 1-2 μm are suitable for flow rates of 20-200 nL/min (nanospray); a 50- μm capillary diameter for flow rates of 0.2 to 4 $\mu\text{L}/\text{min}$ (microspray); and 100-200 μm capillary diameters for flow rates of 1-10 $\mu\text{L}/\text{min}$ [55]. For flow rates in the range 0.1-2.0 mL/min electrospray nebulization alone is inadequate for spray formation. A pneumatic-assisted nebulizer or ionspray, consisting of an electrospray capillary surrounded by a larger coaxial capillary through which flows a high velocity gas is required in this case. The optimum flow rate for pneumatically assisted nebulization is about 50-200 $\mu\text{L}/\text{min}$, although higher flow rates can be used. Higher than optimal flow rates are unnecessary in practice, since the ion current is hardly increased at higher flow rates compared with using a flow splitter at the column-probe connection and independently operating both the column and interface at their optimum flow rates. Heated nitrogen gas is applied as a countercurrent, concurrent or perpendicular flow to the spray, depending on the source design, to enhance droplet desolvation. Off-axis or orthogonal positioning of the capillary sprayer with respect to the sampling orifice, for example Figure 9.5, allows higher flow rates to be used and significantly reduces the contamination of the sampling orifice. Z-Spray represents an alternative design for a high flow rate electrospray interface in which ions are orthogonally extracted from the spray into a sampling cone, while larger droplets and nonvolatile material are collected onto a baffle plate, Figure 9.6. Subsequently, the ions are extracted orthogonally from the expansion behind the sampling cone into the high vacuum chamber for mass analysis.

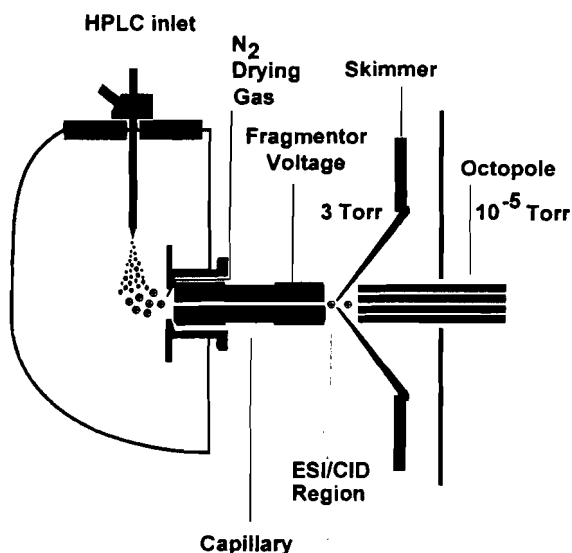


Figure 9.5. Schematic diagram of the Agilent orthogonal electrospray ionization source for LC-MS. (From ref. [29]; ©Elsevier)

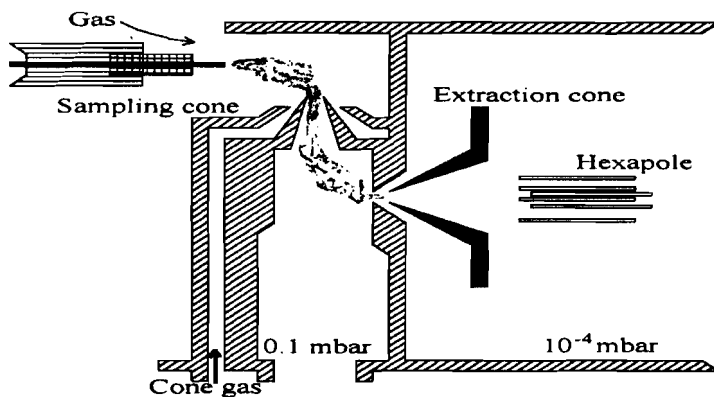


Figure 9.6. Schematic diagram of the Micromas Z-spray electrospray source. (From ref. [54]; ©Elsevier)

Liquid introduction in atmospheric pressure chemical ionization resembles ionspray except that a heated capillary is used in place of the charged capillary, Figure 9.7. The combination of heat and gas flow desolvates the nebulized droplets producing fine particles of solvent and analyte molecules. The solvent molecules are then ionized by a corona discharge and the solvent ions formed ionize the analyte molecules by atmospheric pressure chemical ionization.

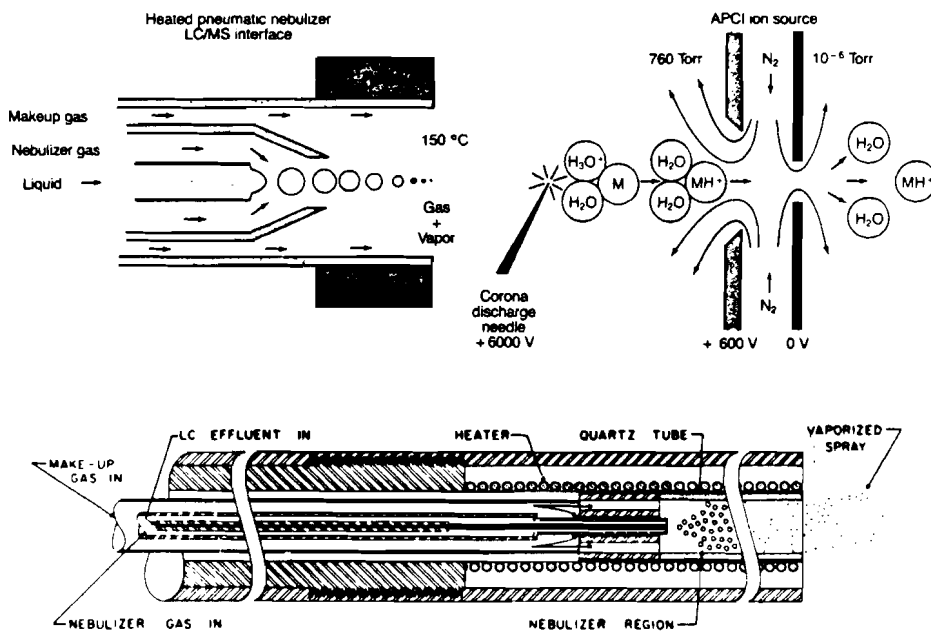


Figure 9.7. Schematic diagram of a heated pneumatic nebulizer LC-MS interface and atmospheric pressure chemical ionization source with a cross-sectional view of the nebulizer probe.

The ions generated together with solvent vapor and nitrogen gas, are desolvated and sampled by an ion-sampling device into a low-pressure region. Desolvation of the droplets is affected by heat and collisions enhanced, either by accelerating the ions through an electric field gradient, Figure 9.5, or by leading them through a gas curtain, as shown in Figures 9.6 and 9.7. Either a heated glass or stainless steel capillary with a potential gradient across its ends, or a conical orifice, is commonly used for ion sampling. The gas flow through the capillary drags the ions towards the vacuum system, even against the electric field gradient. As a result of the transport of ions over a longer distance, the desolvation of ions is assisted. Alternatively, the area in front of the sampling orifice is flushed with nitrogen gas, which acts as a curtain to keep solvent vapors away from the orifice and minimizes clustering of the analyte ions with water and other polar molecules.

The mixture of gas, solvent vapor and ions is supersonically expanded into the low-pressure region. The core of the expansion is sampled by a skimmer into a second region of lower pressure containing an ion focusing and transfer device to optimally focus the ions in a suitable manner for mass analysis. Three-stage differentially pumped vacuum systems are nowadays common on most instruments. The size of the ion-sampling aperture depends on the pumping capacity of the vacuum system and the design of the sampling device, but is generally about 100 μm . For the transfer of ions from the intermediate pressure region to the mass analyzer, a variety of ion optical devices

have been used. Radio frequency-only multipole devices are used in most commercial systems.

The intermediate-pressure region of the ion source has two main functions. It facilitates the transition from atmospheric pressure to high vacuum for mass analysis, and aids in the declustering of solvated ions by ion-molecule collisions. A further possibility is to induce fragmentation by collision induced dissociation. The energy of ion-molecule collisions can be enhanced by applying a small potential difference between the ion sampling aperture and the skimmer, or between the skimmer and multipole ion guide. In this potential field ions obtain sufficient energy for fragmentation to occur in a process known as in-source collision induced dissociation. The purpose is to generate characteristic fragment ions for identification as an alternative to tandem mass spectrometry. It is a simple process to acquire information in this mode, but the reproducibility of the mass spectra among different systems is poor (section 9.2.1.4). A characteristic difference between in-source collision induced dissociation and tandem mass spectrometry is that in the former case all ions present in the ion source are subjected to collision induced dissociation, while for tandem mass spectrometry, only ions of selected mass are dissociated. The fragmentation mass spectra obtained by in-source collision induced dissociation are more likely to be influenced by the presence of contaminants and co-eluting compounds than in tandem mass spectrometry.

The thermospray interface consists of a heated cylindrical tube (ion source) with a vaporizer probe at one end and a pump-out line at the other in a single unit that can be attached to most mass spectrometers with little further modification, Figure 9.8 [1,4,31-33,49]. The vaporizer probe, which connects the liquid chromatograph to the mass spectrometer, contains a resistively heated metal capillary tube (30 cm long and 0.1-0.15 mm internal diameter) that generates a supersonic jet of vapor containing a mist of fine droplets and particles with an input flow rate of 1-2 ml/min. The temperature of the vaporizer is controlled by thermocouples attached to the vaporizer probe. A separate tip heater provides independent control of the tip temperature resulting in improved ion stability. Ions generated in the source are transmitted to the mass analyzer through a sampling cone of 50-75 μm diameter orthogonal to the capillary jet. A repeller electrode placed opposite to the sampling cone is used to improve the ion sampling efficiency. An electron entrance slit and a discharge electrode are placed upstream of the sampling cone to assist in the ionization of samples in nonaqueous or partially aqueous mobile phases containing a high percentage of organic modifier that provide low ion yields by thermospray alone. A mechanical pump with a cold trap is coupled directly to the ion source to provide the additional capacity needed to remove excess vapor delivered to the source by the probe. The successful operation of the thermospray depends on a number of mostly interrelated experimental parameters, of which the most important are the mobile phase composition and flow rate, the source pressure, repeller electrode voltage, and temperature of the vaporizer and source. In addition, it is usually necessary to simultaneously program changes in the vaporizer temperature to obtain maximum ion current throughout a gradient elution separation.

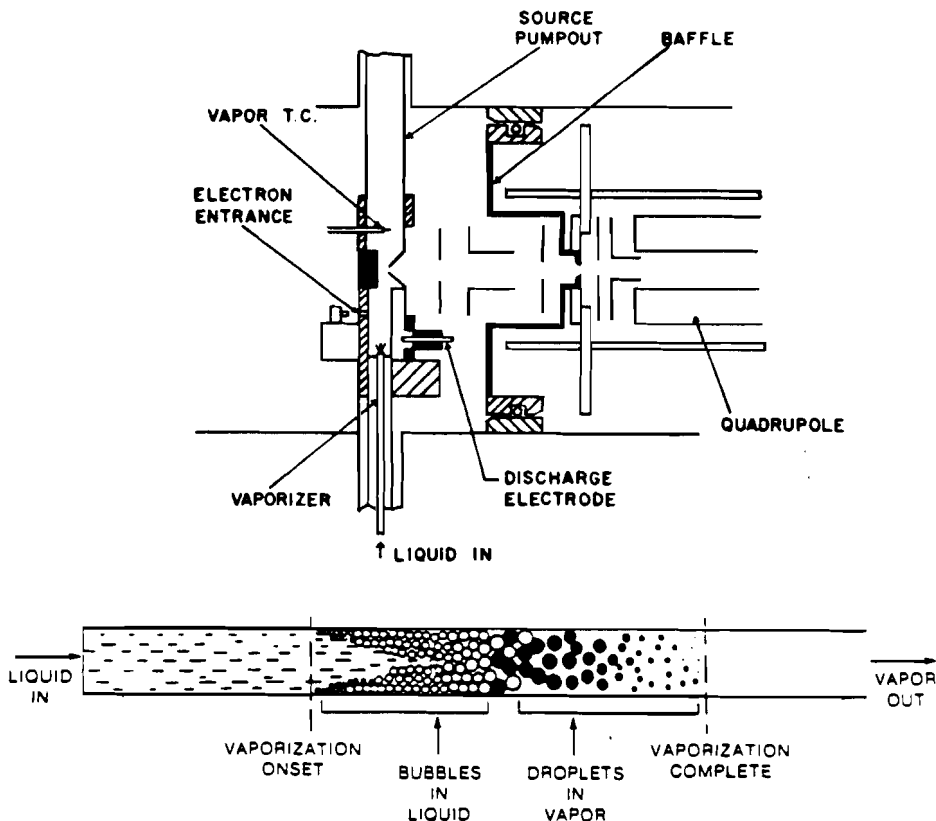


Figure 9.8. Schematic diagram of a thermospray LC-MS interface and insert indicating the mechanism of spray production.

Interfaces for continuous-flow fast atom bombardment are relatively simple although restricted to flow rates of 5-15 $\mu\text{l}/\text{min}$ by the rate of solvent evaporation inside the ion source [1,4,34,60-62]. These flow rates are compatible with separations using packed capillary columns or small bore columns with a flow splitter. The mobile phase is transferred to a target through a narrow fused-silica capillary tube (50-75 μm internal diameter) contained within a probe connected to the ion source by means of a standard vacuum lock. The transfer capillary is terminated at either a stainless steel frit or metal plate. A small percentage of glycerol or other matrix solvent is added to the liquid flow, either at the column exit (coaxial flow probe) or directly to the aqueous component of the mobile phase, to create the correct conditions for the ionization process. The liquid emerging from the capillary forms a thin film over the target, which is bombarded by an externally generated high energy atom (3-8 keV) or ion (< 35 keV) beam producing ions which are subsequently mass analyzed. To aid the continuous renewal

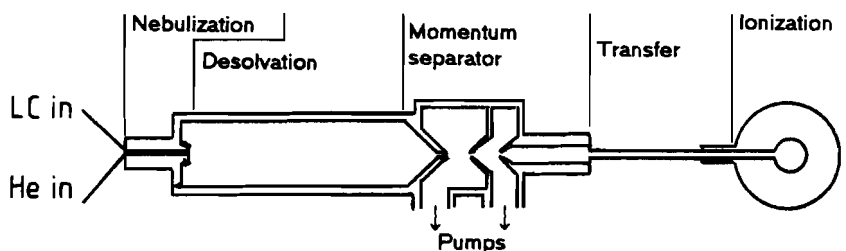


Figure 9.9. Particle beam interface for LC-MS

of sample on the target surface, the liquid flow rate must be stable, and a wick or some mechanical mechanism is employed to accept excess material of low volatility from the target. Factors affecting ion current stability include the wettability of the target, probe temperature, the composition and flow rate of the mobile phase, and selection of the matrix solvent.

Particle beam LC-MS interfaces are unique in that they allow recording of library-searchable electron impact and chemical ionization mass spectra for any compound that is sufficiently stable to enter the gas phase by flash vaporization [49,53,54,63-68]. Since even small traces of solvent vapors interfere in the acquisition of reliable electron impact mass spectra the interface must be capable of complete removal of the mobile phase before the sample is delivered to the ion source. A typical particle beam interface consists of three parts: a nebulizer; a desolvation chamber; and a momentum separator, Figure 9.9. A number of different nebulizers have been described for spray formation, but some form of thermal or ultrasonic assisted concentric pneumatic capillary nebulizer is typically used with dimensions and operating conditions adjusted to accommodate different flow rates from 1-5 $\mu\text{l}/\text{min}$ to 1-2 ml/min . The uniform spray droplets then pass through a heated, low-pressure (e.g. 200-500 Torr) desolvation chamber to promote rapid solvent evaporation. The resulting mixture of submicron solid particles, gas and solvent vapors is drawn into a two-stage momentum separator by the pressure gradient between the desolvation chamber and the ion source. Material in the desolvation chamber is sampled by a small nozzle into a vacuum chamber maintained at about 1-10 Torr. Both the dimensions of the nozzle and the pressure drop result in a supersonic expansion imparting a high momentum to the relatively large solid particles that continue to move as a focused beam in the forward direction. The low-mass solvent and gas molecules acquire limited momentum upon entering the vacuum chamber, and undergo a rapid radial expansion. Separation by mass difference is achieved by sampling the particle beam through a small orifice (aerodynamically shaped skimmer) directly opposite the nozzle, allowing the other components to be removed by the vacuum. The process is repeated to achieve further sample enrichment and pressure reduction in a second vacuum chamber at about 0.1-1 Torr separated from the first chamber by the skimmer. The resulting dry, high velocity particle beam exiting the momentum separator then passes through a short transfer capillary directly into the ion source.

The heated wall of the ion source provides energy for the flash vaporization of the particles and ionization of the gas-phase sample is carried out in the usual way. Full scan, electron impact mass spectra can be obtained from about 10-100 ng of compound. A miniaturized particle beam interface housed entirely within the ion source has been described for eluent flow rates $< 1.5 \mu\text{l}/\text{min}$ [67,68].

Interest in the particle beam interface has declined with the development of more robust and reliable atmospheric pressure ionization interfaces. Thermal stability and volatility requirements limit sample suitability. Memory effects are caused by adsorption of sample or decomposition products on the wall of the source [64]. The build up of foreign material from earlier samples is slowly released by collisions with particles from a new sample producing a variable mixture of ion signals in the mass spectrum. Non-linear mass calibration curves at low sample amounts was attributed to processes occurring in the particle formation step [63,69]. Co-eluting material gives rise to larger particles than those formed by the calibration samples alone. The resulting momentum gain leading to better transport through the separator and hence more material reaching the ion source. Band broadening and tailing during passage through the interface was observed for some compounds [63]. This was attributed to divergence of the particle beam in the transfer capillary and interaction of the sample with the capillary wall.

9.2.2.3 Supercritical Fluid Chromatography-Mass Spectrometry

Common interfaces for supercritical fluid chromatography-mass spectrometry (SFC-MS) are typically LC-MS interfaces with minor modifications to accommodate different column flow rates, mobile phase compositions, and operating conditions of supercritical fluid chromatography [49,70-74]. Atmospheric pressure ionization interfaces are preferred, since these allow the chromatograph and mass spectrometer to operate independently without significant compromises. Also, adjustments to the interface are easily made. Atmospheric pressure ionization interfaces have virtually rendered obsolete particle beam, thermospray, and moving belt interfaces for SFC-MS [49,73]. The low flow rates typical of open tubular column supercritical fluid chromatography ($1\text{-}5 \mu\text{l}/\text{min}$ as liquid or a few ml/min as gas at STP) are compatible with direct fluid introduction using electron impact and chemical ionization sources with adequate pumping capacity. The virtual reliance on carbon dioxide as the only common mobile phase for open tubular column supercritical fluid chromatography is an advantage for direct fluid introduction. Higher mobile phase flow rates (up to $1.5 \text{ l}/\text{min}$ as gas at STP) together with solvent modifiers and composition gradients are typically used for packed column supercritical fluid chromatography. An enrichment interface is required for these conditions.

The direct fluid interface consists of a probe housing a portion of the column or transfer line with a restrictor at the tip. The functions of the restrictor are to maintain the sample in solution throughout the interface, to control the flow rate through the column, and to provide a mechanism to release the fluid into the ion source as a gas. The critical features of the interface are that: it must maintain the column oven temperature

up to the restrictor; provide an additional heat source at the restrictor tip; provide low dead volume connections throughout the interface; and allow exact positioning of the restrictor tip in the active region of the source volume. The restrictor is separately heated to compensate for cooling during the fluid expansion to the vapor phase and to diminish the formation of solvent clusters. The higher pressures of chemical ionization sources also helps to minimize cluster formation.

Ionization occurs by both charge exchange with CO_2^+ ions and by electron bombardment in conventional electron impact ion sources using the direct fluid interface. This is not usually a great disadvantage, since the mass spectra produced resemble electron impact spectra that can be searched in mass spectral libraries. Although charge exchange mass spectra generally exhibit less fragmentation on account of the mild ionization conditions. The relatively high ion source pressures and its variation during density programming can lead to poor and variable sensitivity. Under these circumstances, the electrons generated by the filament have a shorter penetration range, reducing significantly the ionization efficiency by electron bombardment and charge exchange. The ion transmission efficiency may also be adversely affected. For chemical ionization, reagent gases are introduced into the source by a separate inlet. The reagent gas usually suppresses charge exchange ionization by carbon dioxide producing predominantly molecular ion adducts by proton exchange or nucleophilic addition, depending on the reagent gas. Common problems with direct fluid introduction interfaces are restrictor plugging, pressure dependent changes in mass spectrometer performance during density-programmed separations, the low sample capacity of open tubular columns, and the limited solvating power of supercritical fluid carbon dioxide for polar compounds. Some of these problems disappear with the use of packed columns and atmospheric pressure ionization interfaces.

Generally, standard LC-MS atmospheric pressure ionization interface probes with nebulization gas and makeup flow capabilities are used for both packed and open tubular column supercritical fluid chromatography after relatively minor modification [74-79]. For open tubular columns the flow restrictor is located at the tip of the nebulizer and heated by the flow of nebulizer gas or by a separate heater. Either a backpressure regulator or regulated pressurized liquid flow and postcolumn tee piece is used to provide flow control for packed columns. With mixed mobile phases and composition programming, it is important that the column outlet pressure is high enough to ensure that the mobile phase remains a single phase up to the expansion point, as illustrated in Figure 9.10. Adding the pressure-regulating liquid at a postcolumn tee provides a low-dead volume method of regulating pressure, as well as enriching the mobile phase in the polar organic solvent during the pressure reduction step. This ensures adequate solubility of the sample throughout the interface as temperature variations are encountered. The pressure regulating liquid is usually the same as the mobile phase modifier to avoid possible problems with phase separation. A coaxial sheath-flow liquid is added at the middle tee and flows in the space between the electrospray capillary and a larger fused-silica capillary combining with the mobile phase at the tip of the spray device. Nebulizer gas is added at the sprayer tee. Sheath-flow pneumatically assisted

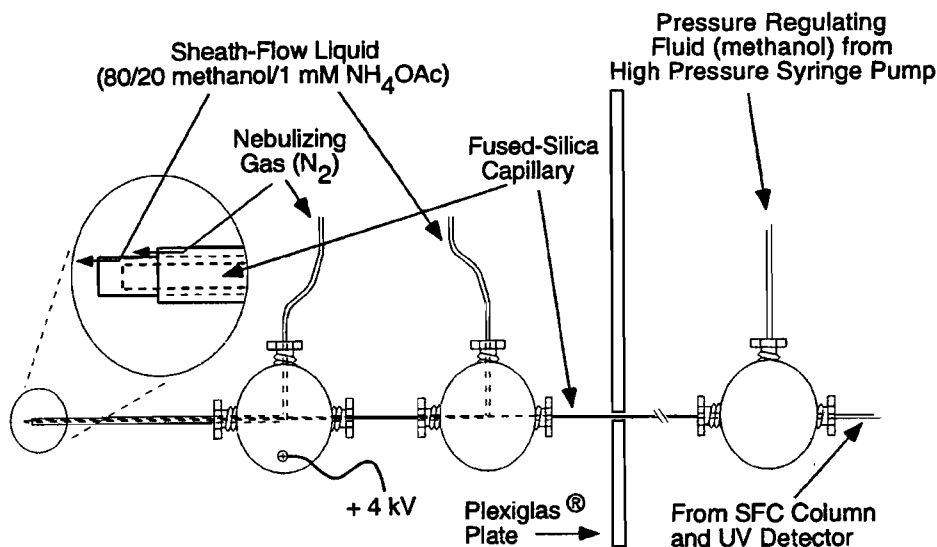


Figure 9.10. Schematic diagram of a packed column SFC-MS interface with electrospray ionization. This interface features a liquid pressure-regulating flow for pressure control to just before the expansion region. (From ref. [75]; ©Elsevier).

electrospray eliminates the influence of the mobile phase composition on the ionization process and eliminates tailing of low volatility compounds that otherwise accumulate on the electrospray capillary. The sheath-flow liquid is usually an aqueous organic solvent containing a low concentration of a volatile buffer, such as ammonium acetate, and provides enhanced and more uniform ionization conditions.

The criteria for optimum performance of atmospheric pressure chemical ionization and electrospray are different. For electrospray, it is important to control the flow rate and composition of the sheath-flow liquid, the mobile phase flow rate, and the dimensions of the electrospray and sheath-flow capillaries, as well as their relative position with respect to each other. Sample ionization with carbon dioxide alone does not occur in the absence of sheath-flow liquid. For atmospheric pressure chemical ionization, apart from those factors that affect the performance of the nebulizer, the flow dynamics throughout the interface and temperature in the ionization region, are the most important parameters. Moistened air is sometimes used as a makeup gas to maintain stable operation of the atmospheric pressure chemical ionization interface with H_3O^+ ions supplementing solvent ions in the reaction gas plasma. The ionization efficiency of both methods is similar to liquid chromatography.

9.2.2.4 Capillary Electrophoresis-Mass Spectrometry

Capillary-electromigration separation techniques are coupled to mass spectrometry (CE-MS) by interfaces based on proven LC-MS designs [41,49,80-85]. In early

studies continuous-flow FAB interfaces were used [81], but now virtually all reported applications employ electrospray interfaces. All capillary-electromigration separation techniques can be coupled to mass spectrometry in a similar manner, although reported practical applications are dominated by capillary electrophoresis. Singular problems with micellar electrokinetic chromatography (MEKC) mean that there are no routine applications of MEKC-MS at present. Typical surfactants added to the separation buffer for micelle formation in micellar electrokinetic chromatography are nonvolatile and reduce the ionization efficiency of electrospray, contaminate the interface, and produce mass spectra overwhelmed by low molecular mass background ions. There is no comprehensive solution to these problems. Limited success has been obtained using high molecular mass surfactants [86], removal of surfactant at the end of the capillary in a membrane separator [87], and the partial filling technique [88]. Coupled column systems and voltage switching for transfer of the compounds of interest from the micellar electrokinetic chromatography separation column to a second column containing an electrolyte solution without surfactant connected to the mass spectrometer, offers an alternative approach [89]. The partial filling technique is the most popular approach, but all these methods are at best awkward and none are compatible with high sample throughput methods. The partial filling technique requires that only the injection end of the capillary is filled with surfactant solution, in which sample separation occurs, and the detector end of the capillary is filled with an electrolyte solution that is responsible for separation of the sample from the surfactant.

The adaptation of LC-MS interfaces for CE-MS must consider general differences in the typical operating conditions for capillary electrophoresis compared with liquid chromatography. Typical flow rates in capillary electrophoresis are less than 100 nl/min. These low flow rates are incompatible with widely available low flow rate LC-MS interfaces, which preferentially operate in the range of 1-50 μ l/min. A makeup buffer and/or miniaturized electrospray system is required to obtain optimal ionization efficiency and efficient sample transport to the ion source. The small peak volumes require low-dead volume connections throughout the interface, and appropriate mass spectrometer scan rates. Many of the common low volatility buffers used in capillary electrophoresis are generally unsuitable for CE-MS. They often suppress sample ionization and contribute significantly to the chemical background. When compatible with separation requirements, volatile buffers at low concentrations are preferred. The interface must also provide an appropriate mechanism for maintaining electrical control of the separation at the column exit. The electrical control provides the return path for both the separation column and the electrospray current flow. The low loading capacity of capillary-electrophoretic separation techniques is a general disadvantage in terms of concentration sensitivity. Sample concentrations of about 10^{-5} M are generally required to obtain good quality mass spectra.

Either a sheathless, coaxial sheath-flow, or liquid-junction interface is commonly used for CE-MS, Figure 9.11. The reproducible and straightforward construction of the coaxial sheath-flow interface has resulted in its general use, although the sheathless interface provides higher sensitivity. The difficulty in making low-dead

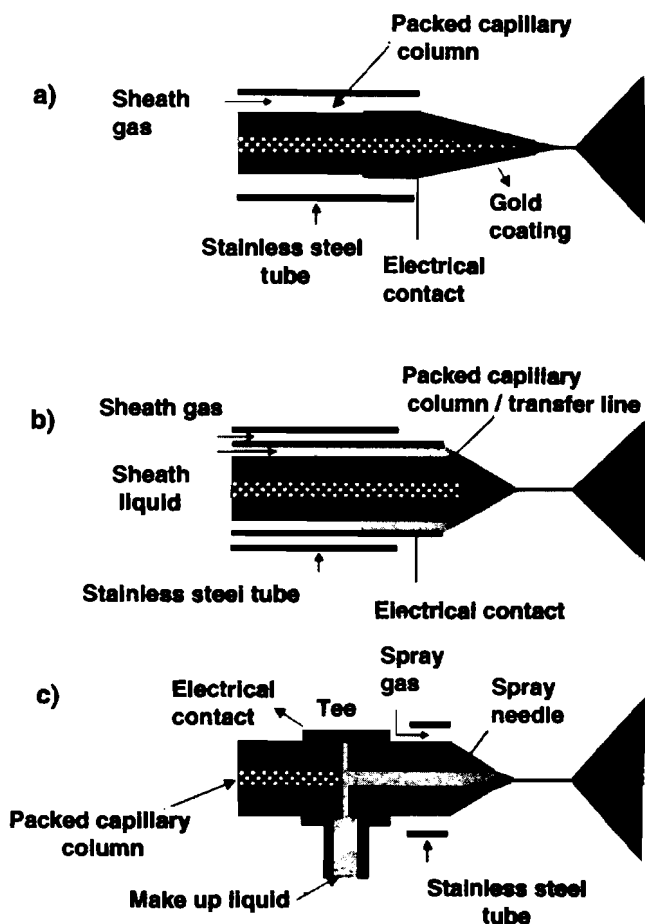


Figure 9.11. Schematic diagram of (a) sheathless, (b) sheath-flow and (c) liquid-junction electro spray interfaces for CE-MS. (From ref. [84]; ©Elsevier).

volume connections and aligning the separation capillary with the transfer capillary has contributed to the decline in use of the liquid-junction interface. Nanospray and microspray ionization techniques are a natural choice for low flow electro spray [83-85,90-94]. First, the close proximity of the spray tip to the ion sampling orifice results in a larger proportion of the ions being transferred to the vacuum system of the mass spectrometer. Secondly, at low flow rates smaller droplets are formed which aids ionization. Lastly, nanospray and microspray require less or no makeup liquid flow compared with conventional electro spray, resulting in higher sensitivity.

In the sheathless interface the tip of the capillary column is the spray point. The tip is pulled out or sharpened to a fine point with an orifice of about 5-20 μm . The outer

surface of the capillary tip is covered by a film of metal particles, or a cured resin containing conductive particles (e.g. gold, silver, or carbon). Poor mechanical stability and poor resistance to oxidative stress result in limited lifetimes for metal coatings compared with the mixed polymer/particle coatings. The conductive coating serves as the electrical contact for the separation voltage and spray voltage. A few manufacturers offer metal-coated capillary tips that can be butt-connected to the separation capillary. A sheath gas, such as SF₆ is often used with these interfaces to prevent corona discharge due to the high field strengths present at the sprayer tip. The small components of the nanospray interface are difficult to manipulate, but as compensation zone broadening in the interface is minimal, and an increase in sensitivity of from 10-50 fold compared with a conventional electrospray interface is obtained.

In the coaxial sheath-flow interface the separation capillary protrudes slightly from a larger bore metal capillary tube, which delivers a sheath liquid to the column exit. Electrical contact with the metal capillary completes the circuit for the separation column and provides the voltage for electrospray. A third concentric tube may deliver a gas flow to assist in spray formation. The distance between the separation capillary and sheath tubing should be minimized to provide a stable electrospray ionization current and a small mixing volume at the tip to minimize band broadening. The column flow is usually less than 0.1 $\mu\text{L}/\text{min}$ while the sheath-liquid flow is substantially higher at about 1-10 $\mu\text{L}/\text{min}$. In this way the electrospray process is dominated by the sheath liquid and the limitations on the selection of the separation buffer may be relaxed. The sheath liquid also acts as the outlet reservoir for the separation column, and frequently has a different composition from the inlet buffer reservoir. The properties of the sheath liquid are selected to improve electrospray performance, for example, organic solvents are added to lower surface tension and volatile weak acids to enhance the ionization efficiency. Under certain conditions, ions from the sheath liquid may diffuse into the separation capillary and alter the migration time and migration order of the sample.

The liquid-junction interface consists of a metal tee with an electrical connection and outlet buffer reservoir. The ends of the separation column and electrospray capillary are positioned opposite each other at the center of the tee with a gap between them of 10-25 μm . The outlet reservoir provides additional makeup flow to the separation buffer through the third branch of the tee. The interface decouples many aspects of the separation system from the electrospray source and can be used with a wide range of column flow rates. On the other hand, it is rather cumbersome to use, particularly the alignment of the capillaries. In addition, it is difficult to avoid band broadening at the open connection and the makeup liquid dilutes the sample concentration at the sprayer.

9.2.2.5 Thin-Layer Chromatography-Mass Spectrometry

The problems to be solved in interfacing thin-layer chromatography to mass spectrometry (TLC-MS) are largely different to those experienced in LC-MS and other column separation techniques [95-99]. At the completion of the separation the chromatogram is fixed in time and space with the major portion of the mobile phase eliminated by evaporation. The thin-layer plate can be considered as a storage device effectively de-

coupling the separation requirements from those of measuring the mass spectra. The immobilized separation is also generally compatible with the vacuum requirements of the mass spectrometer. On the other hand, the sample is embedded within the stationary phase layer from which it must be extracted into the gas phase and ionized for mass analysis. It is perhaps not surprising, therefore, that the principal methods of instrumental TLC-MS are based on surface desorption and ionization techniques using fast atom bombardment [97,99-101], laser desorption and ionization [102,103], and MALDI [40,99,104-107]. Commercial interfaces provide limited automation capabilities. Only in the literature are systems providing automated plate scanning, chromatogram recording, and mass spectrum acquisition under software control for single isolated tracks, and less commonly with full plate scanning capability, described. Fortunately, manual methods based on excising sample zones from the plate followed by thermal, laser, or fast atom bombardment for desorption from the sorbent matrix in the ion source, or similar treatment of sample residues obtained by solvent extraction from the sorbent, are simple and straightforward to carryout, if tedious [96,98,99,108]. As an alternative to these general approaches, a probe for direct solvent extraction of individual separated zones followed by electrospray ionization of the extracted sample has been described [109].

Fast atom bombardment mass spectra are routinely obtained by plate scanning or simply using a strip of doubled-sided sticky tape to attach excised areas of the chromatographic zone to a standard probe. A few μl of matrix solution is added to the sorbent, and the probe introduced into the mass spectrometer to acquire spectra in the usual way. Alternatively, the sample zone can be concentrated by scribing a trapezoidal shape around the sample zone followed by elution with a strong solvent to migrate the sample to the tip of the trapezoid. The sorbent containing the concentrated sample is then removed and mixed with matrix solvent for introduction into the FAB source. In scanning methods the dried plate, or a portion of the plate, is fixed on a translation stage and moved at a constant velocity through the ion beam in the evacuated FAB ion source. To improve the sensitivity of the method, a matrix solution or phase transition matrix is applied to the layer before spectral acquisition and time averaging of the ion signal employed. Because ions are removed from the surface only, the ionization efficiency will be low unless a mechanism is provided to extract and cycle the major portion of the sample to the surface in a continuous manner. Impregnating the chromatogram with a viscous liquid or low melting point solid (phase transition matrix) fulfills this role without destroying the integrity of the chromatogram through zone broadening. By using a phase transition matrix a steady signal can be obtained for several hours permitting extended data collection. For reliable mass spectra in the scan mode sample sizes in the tens of nanograms to microgram range are required. Since FAB ionization produces largely molecular ion adducts with limited fragmentation, tandem mass spectrometry and collision induced dissociation are used for confirmation of identity [97,99-101].

Methods relying on MALDI require application of an extraction solvent to the layer to move sample to the layer surface followed by co-crystallization with a MALDI matrix material. TLC-MALDI direct coupling methodologies use one of three methods of layer

treatment. A MALDI matrix solution is deposited directly on the layer, or better still, applied to the layer by electrospray, and the solvent evaporated with crystallization of the matrix [106,107]. The main problem with this approach is loss of separation integrity due to zone broadening caused by convection driven by matrix crystallization. The pressing method attempts to reduce sample spreading by separating the extraction and crystallization steps [104]. A MALDI matrix layer is formed on a smooth inert substrate, separate from the TLC plate, and is transferred to the surface of the separation layer by pressing the matrix layer and TLC plate face-to-face in the presence of a sprayed-on extraction solvent. The spatial resolution and detection limits are largely determined by the selection of the extraction solvent, the extraction time, the pressure and time used for the pressing step, and the thickness of the stationary phase. Even for optimum conditions, the sample recovery remains low (< 22 %) because of the poor extraction efficiency of the sample from the sorbent pores, as well as some lateral broadening of sample zones. The extraction efficiency is virtually complete using a hybrid TLC-MALDI plate, in which two juxtaposed layers, a separation layer and a MALDI matrix layer, are formed on a common support. The separation is performed in one direction on the TLC layer, the mobile phase evaporated and the MALDI matrix applied to the MALDI zone, followed by elution of the sample in the direction of the MALDI matrix layer [105]. The MALDI matrix layer is used to acquire the mass spectra. Optimization of all three methods is critical to their success, with good quality mass spectra obtained from picogram to nanogram amounts of sample.

TLC-MS is generally used for measuring spectra and is not considered reliable for quantitative analysis, for example, by selected ion monitoring. For low molecular mass compounds, spectral quality may be compromised by the chemical background resulting from ionization of matrix materials, the binder used in preparing the layers, and general contaminants adsorbed by the layer during storage and use.

9.2.3 Mass Analyzers

Ions leaving the ion source must be separated according to their mass-to-charge ratio (m/z) prior to detection. This is achieved by imposing external electric or magnetic fields on the ion beam to affect a separation of the ions in time or space. Important properties of a mass analyzer are its mass range, mass accuracy, ion transmission properties, scan speed, and mass resolution, Table 9.4 [1-6,110,111]. The resolving power of a mass spectrometer is a measure of its ability to distinguish between two neighboring masses. In general, mass spectrometers operate in two resolution regimes, low to medium resolution with values of 500-3,000, and high resolution with values of 10,000-150,000. High-resolution measurements provide exact elemental compositions for all selected ions in the mass spectra and require more complex instrumentation (section 9.2.3.6). Low-resolution mass spectrometry is routinely used for chromatographic applications with magnetic sector, quadrupole mass filter, ion trap, and time-of-flight mass analyzers. A number of considerations are involved in selecting a mass analyzer for laboratory use, including extrinsic factors such as cost, size, ease of use, and compatibility

Table 9.4

Representative mass analyzer performance characteristics

Property	Magnetic sector	Quadrupole mass filter	Ion trap	Time-of-flight	FT ion cyclotron resonance
Resolution ($M/\Delta M$)	10^2 - 10^5	10^2 - 10^4	10^3 - 10^4	10^3 - 10^4	10^4 - 10^6
Mass accuracy (ppm)	1-5	100	50-100	5-50	1-5
Upper mass range	10^4	10^4	10^5	$> 10^5$	$> 10^4$
Linear dynamic range	10^9	10^7	10^2 - 10^5	10^2 - 10^6	10^2 - 10^5
Precision (%)	0.01-1	0.1-5	0.2-5	0.1-1.0	0.3-5
Abundance sensitivity	10^6 - 10^9	10^4 - 10^6	10^3	up to 10^6	10^2 - 10^5
Efficiency (%)	< 1	< 1 -95	< 1 -95	1-100	< 1 -95
Speed (Hz)	0.1-20	1-20	1-30	10 - 10^4	0.001-10
Cost	Moderate	Low	Low	Moderate	High
Size	Floor standing	Benchtop	Benchtop	Benchtop	Floor standing

with different chromatographic inlets, as well as analyzer performance characteristics. The upper limit of the m/z range may be crucial for some applications involving biopolymers, for example, but of little consequence for gas chromatography where the molecular mass elution range falls within the range available for most mass analyzers. The range over which the ion signal is linear with sample concentration (linear dynamic range) is of interest in quantitative analysis when the concentration of sample ions differ significantly or target ions are present at very different concentrations in a series of samples. The abundance sensitivity (inverse of the ratio obtained by dividing the signal level associated with a large peak by the signal level one m/z unit lower or higher) is an important consideration for isotope ratio measurements, but less important for spectral acquisition. Acceptable precision (the reproducibility with which ion abundance can be determined) is required for recording mass spectra for reference purposes. Speed is a measure of the number of spectra that can be recorded in unit time. It is an important factor for fast chromatography (section 9.2.3.5) where rapidly eluting peaks may preclude accurate measurement of a mass spectrum by averaging a sufficient number of transient spectra across the peak width.

9.2.3.1 Magnetic Sector

For mass analysis, ions are accelerated into an evacuated and curved flight tube positioned between the pole pieces of an electromagnet. If the ions are accelerated by a voltage V (2-8 kV) into a flight tube of radius r , then ions of the same mass-to-charge ratio (m/z) are brought to focus at a point according to the relationship $m/z = (H^2 r^2 e)/2V$, where H is the magnetic field strength and e the charge of an electron. The complete mass spectrum is recorded by scanning, that is, sequentially bringing all ions of different m/z into focus at the detector. The most common form of mass scan is the exponential magnet scan, downward in mass, in which V is kept constant and H is varied in time. This scan mode has the advantage of producing constant ion peak widths throughout the mass spectrum. The alternative of changing V over

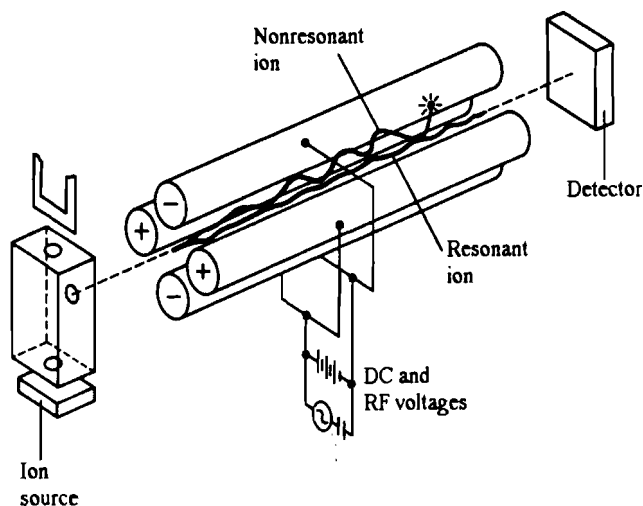


Figure 9.12. Schematic diagram of a quadrupole mass filter.

a wide range to collect an entire spectrum at a constant magnetic field strength leads to a defocusing of the ion beam with a loss of resolution and lower sensitivity for high m/z ions. Narrow voltage scans, however, are convenient for rapid switching between defined m/z values for selected ion monitoring. Scan times of at least 1-2 s per decade of mass (e.g. 50-500 Da) are required to accurately record the mass spectrum of peaks in open tubular column gas chromatography. This is possible with modern laminated magnets, but hysteresis eventually limits scan speed, and magnetic sector instruments are not suitable for fast chromatography. Magnetic sector instruments are generally more expensive and complex to use than the mass analyzers described below. Also, the high voltages required for ion acceleration are incompatible, or difficult to implement, with some ion sources used with chromatographic sample inlets. For these reasons (and others) magnetic sector instruments have declined in use for general-purpose instruments, but remain a common component of high-resolution instruments for accurate mass measurements and for physicochemical studies of energetic ions.

9.2.3.2 Quadrupole Mass Filter

The quadrupole mass filter consists of (usually) four parallel hyperbolic or cylindrical rods in a square array, Figure 9.12 [1-4,110-113]. Opposite rods are electrically connected to a voltage supply made up of a direct current component, U , and a radio frequency component, $V\cos(\omega t)$, where V is the radio frequency voltage and ω its frequency. The voltages applied to each set of rods have opposite signs but equal magnitude. Ions entering the mass analyzer are made to oscillate in the field produced by the rods in a complex fashion. The motion of an ion in the electric field depends on its mass-to-charge ratio, the voltages U and V , and the frequency of the radio

frequency potential. At any given moment, only ions of one particular mass-to-charge ratio have stable trajectories through the quadrupole mass filter and reach the detector. All other ions will oscillate with greater amplitudes, causing them to become unstable and neutralized through collision with one of the rods. To scan the mass spectrum, the frequency of the radio frequency voltage and the ratio of the alternating current/direct current voltages are held constant, while the magnitude of the alternating current and direct current voltages are varied. The transmitted ions of mass m/z are then linearly dependent on the voltage applied to the quadrupoles, producing a m/z scale that is linear with time. The voltages applied to the rods are usually chosen to give equal peak widths over the entire mass range and unit resolution (resolution sufficient to separate two ions one mass unit apart) throughout the mass spectrum.

Quadrupole mass spectrometers are more compact, inexpensive, and easier to operate than magnetic sector instruments. Electric fields are easier to control than magnetic fields facilitating the tuning of chosen m/z values for selected ion monitoring. Quadrupole mass filters are somewhat more tolerant of high source pressures because of the relatively low voltages applied to the rods, and can be rapidly pulsed to facilitate simultaneous recording of positive and negative ion mass spectra. This is not possible with magnetic sector instruments. For a given radio frequency voltage and frequency, the length and diameter of the rods determine the mass range and maximum resolution that can be achieved by the quadrupole assembly. The upper mass limit that is normally achieved, however, is around 600-4000 Da (depending on design) with a resolution of around 3000.

The introduction of low cost, benchtop mass selective detectors based on the favorable operating characteristics of quadrupole mass filters, was largely responsible for the rapid acceptance of the mass spectrometer as a routine chromatographic detector. Mounting the ion source, quadrupole mass filter, and ion detector within the diffusion pump housing of the mass spectrometer vacuum system enabled compact instruments to be designed [114,115]. The whole instrument is operated by a desktop computer, which performs control functions as well as a full array of data processing tasks. The quadrupole mass selective detector was soon followed by ion trap detectors with similar virtues of low cost, ease of use, and compact design, as well as providing a low-cost entry to MS^n experiments, otherwise available only on triple quadrupole and similar instruments (section 9.2.4) [116-118].

9.2.3.3 Ion Trap

Ion formation, storage, and scanning can be performed in the same chamber with the ion trap, although most systems in current use employ an external ion source and a pulse mechanism to introduce ions into the trap [115,117-123]. Although the ion trap is based on the quadrupole mass filter, its orientation is completely changed, Figure 9.13. The radio frequency signal controlling scanning, instead of being applied to the conventional rods, is now applied to a central, circular ring electrode situated between two end-cap electrodes held at appropriate potentials. Ions are stored in the ion trap by application of a radio frequency voltage to the ring electrode. Stable orbits, in which ions are trapped

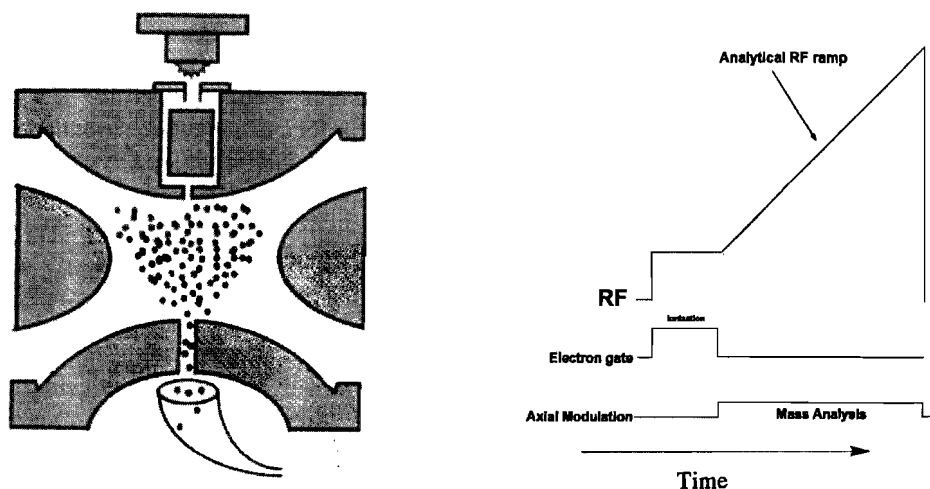


Figure 9.13. Schematic diagram of an ion trap mass analyzer with scan function for electron impact ionization and axial modulation for mass analysis. (From ref. [121]; ©John Wiley & Sons).

in the cell, correspond to regions of stable motion in both the axial and radial directions. The ion trap is operated at the comparatively high pressure of 10^{-2} to 10^{-3} Torr to dampen the motion of the stored ions, and prevent their immediate loss to the end caps. Collisions with helium atoms reduce the kinetic energy of the trapped ions causing their migration towards the center of the trap.

A unique feature of the ion trap is the use of an automatic gain control to simultaneously minimize the influence of space charge effects at high sample concentrations, and to maximize sensitivity at low sample levels. Too many ions in the trap can result in distortion of the mass spectrum through collisions. The automatic gain control adjusts the ionization time for each scan according to the product of the sample size and ionization efficiency, to avoid overloading the trap at high sample concentrations, while maximizing the number of ions in the trap at low sample concentrations. This is accomplished by a preliminary short scan of about 1 ms in which all ions above a certain threshold value are collected. From this preliminary scan the full scan acquisition time is estimated and the ionization time set.

After setting an appropriate radio frequency voltage at the ring electrode, the ions are injected as a pulse from the external source and stored in the trap. For selected ion monitoring, all ions other than the selected ion are ejected from the trap. Any trapped ion can be fragmented by collisions with gas atoms and resonant radio frequency energy stimulation, with all product ions stored in the trap. The product ions can then be analyzed by resonant expulsion. Thus time-dependent MS^n experiments are easily performed in this way with results that are similar, although not necessarily identical, to space dependent tandem mass spectrometry (section 9.2.4). The process can be repeated several times, selecting successive fragment ions, and the value for n can be quite large

and only limited by ion depletion. In the full-scan mode, ions of different mass-to-charge ratio are consecutively ejected from the trap by resonant expulsion. A new AC potential is superimposed on the radio frequency potential and tuned to the ion characteristic frequency. This increases the energy of the ions whose motion eventually corresponds to unstable trajectories in the axial direction. As a result, and in order of increasing mass-to-charge ratio, these ions leave the trap through holes in one of the end-cap electrodes and strike the detector. The main disadvantage of the ion trap is that the pressure inside the trap has a crucial influence on spectral quality.

9.2.3.4 Time-of-flight

Early time-of-flight (TOF) mass analyzers suffered from poor mass resolution and were little used with chromatographic sample inlets. This situation changed completely in the 1990s with the development of the reflectron and the orthogonal accelerator, and received further impetus from advances in microelectronics and computers [124-130]. TOF mass analyzers are ideally suited for use with pulse ionization methods, such as MALDI (section 9.2.1.7), and initially it was the rapid acceptance of MALDI-TOF for the analysis of biopolymers that accelerated interest in TOF mass analyzers. Today, TOF mass analyzers are seen to have a number of advantages when used alone or as a component of hybrid instruments (section 9.2.4). This has promoted rapid growth in their applications with chromatographic sample inlets.

For a simple picture of mass analysis by time-of-flight, a pulse of externally produced ions is accelerated to a constant kinetic energy and allowed to drift through an evacuated field free flight tube. The ions acquire a characteristic drift velocity, which is proportional to the square root of their mass-to-charge ratio, and arrive at the detector separated in time. Thus, the mass-to-charge ratio, m/z , for the ions is determined from a measurement of the time, t , taken to traverse a specified flight path, L , according to $t^2 = mL^2/2zeV$, where e is the charge on an electron and V the initial acceleration voltage. To obtain a mass spectrum, all that is required is that the number of ions arriving in a defined time window is detected. Mass resolution is compromised, however, by the kinetic energy spread of the ions before acceleration that results in ions of the same mass arriving at the detector at slightly different times.

The spread of ion kinetic energies can be minimized using a TOF mass spectrometer fitted with a reflectron and/or an orthogonal accelerator, Figure 9.14. The reflectron is an ion mirror consisting of a series of grids, which provides a compensation of flight-time differences for ions with the same m/z by stopping them in an electric deceleration field and reversing their flight direction before detecting them. Higher-energy ions of the same m/z penetrate deeper, and spend more time in the device, than their lower-energy counterparts. The result is that the spread in drift times for ions of the same m/z is reduced and resolution improved.

For ions produced in the gas phase, typical of most ion sources coupled to separation systems, a combination of quadrupole injection and an orthogonal accelerator produces a focused ion beam with a small kinetic energy distribution from virtually any ion source. Early interfaces between the ion source and mass analyzer merely provided

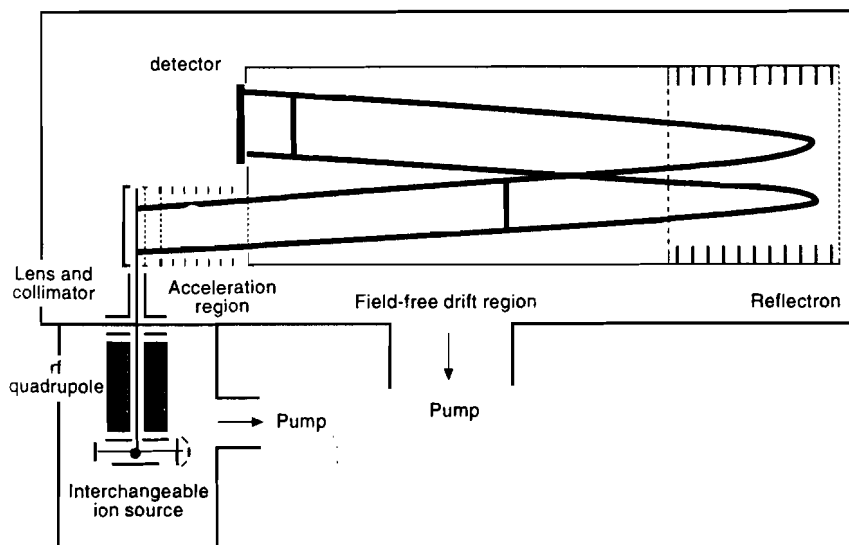


Figure 9.14. Schematic diagram of a typical orthogonal accelerator time-of-flight mass spectrometer with reflectron and radio frequency-only quadrupole ion injection. (From ref. [128]; ©American Chemical Society).

collimation to define the beam shape and differential pumping to reduce pressure from the source region down to that of the analyzer region. Direct injection is limited by the spatial distribution of ions within the source that cause a considerable loss of ions upon collimation and the velocity distribution of the ions in the direction of separation results in a decrease in resolution. It is possible to overcome these limitations by collision damping of the ions in a radio frequency-only quadrupole ion guide at relatively high pressures. The radio frequency field causes the ions to oscillate while gas collisions reduce their velocities to near thermal values. This produces an ion beam with a small spatial spread in the separation direction and a small-radial velocity distribution, and these properties are almost independent of the original parameters of the beam delivered by the source.

The orthogonal accelerator is suitable for use with continuous ion sources that hitherto had proven difficult to interface to a TOF mass analyzer because of ion sampling efficiency limitations imposed by the need to pulse the source or gate the ion beam. The ion source continuously injects a nearly monoenergetic ion beam into the storage region of the orthogonal accelerator, between a flat plate and a grid. When a voltage pulse is applied to the plate, an ion packet is pulsed out of the storage region in a direction nearly parallel to the separation axis, and accelerated to an energy of several keV by a uniform electric field in the second stage of the accelerator. Normally, the voltage pulses of several kV are applied at a frequency of a few kHz up to 40 kHz. An axially symmetric ion beam will contain ions with a zero average velocity component in the direction

orthogonal to the beam. Thus, although the ions may possess a significant kinetic energy distribution, the energy spread will not affect the time it takes ions accelerated in the orthogonal direction to drift to the detector.

The orthogonal accelerator is not a storage device. The beam simply fills the orthogonal accelerator in about the time it takes for the accelerated ion of highest m/z to reach the detector in the TOF analyzer. In the penetration or “fill-up mode”, the first stage of the accelerator is set to be field free. Once the ion beam has “filled the orthogonal accelerator”, a field is rapidly generated in the first stage to accelerate the ions orthogonally relative to their velocity in the beam. Ion arrival at the detector is timed from the gating event. Spectra are averaged and produced at a rate of 10 to 500 per second.

Time-of-flight mass analyzers have a number of attractive features. They produce a complete mass spectrum from each ion-gating event combined with a mass range limited only by the detector's ability to convert the arrival of an ion into a measurable electrical signal. The technology is simpler compared to scanning analyzers with few ion optical elements. Ion transmission is high and sensitivity, particularly in the full-scan mode, is improved, since they do not scan but detect the whole mass spectrum at the same time. Time-of-flight mass analyzers are also ideal as the second stage in hybrid mass analyzers capable of accurate mass measurements when using chromatographic sample inlets.

9.2.3.5 Scan Rates

Each type of mass spectrometer has a maximum scan rate, which is determined by the fundamental processes governing the scan function and the minimum time required to measure the intensity at each mass. Most modern mass spectrometers can be scanned at 10-20 mass spectra per second, which is sufficient for the majority of chromatographic applications, that is separations measured in minutes and peaks a few seconds wide [41,131]. In order to minimize distortions of the ion intensity in the mass spectrum owing to changes in sample concentration in the ion source during a single scan, spectra should be recorded at a rate of at least 5-10 spectra per peak width. Similar scan rates are required for accurate reconstruction of the chromatogram from consecutively recorded mass spectra, and to take advantage of mass-based deconvolution software for resolving overlapped chromatographic peaks. Deficiencies in scan rates are observed for fast chromatography (section 1.7) and some high-resolution separations in gas and capillary electrophoresis.

Spectrum acquisition rates are a major concern in high-speed GC-MS where total separation times in seconds and peak widths typically less than 200 ms are common. The separation conditions are relatively easy to achieve with short, wide bore, open tubular columns operated at low pressures [131-136]. The ion source of the mass spectrometer provides the vacuum source for the column. Time-of-flight mass analyzers are most suitable for these applications. Time-of-flight mass spectrometers in production are capable of recording up to 500 full-mass spectra per second based on averaging 10 transient spectra per single spectrum [135-138]. At present, this is a unique

application of time-of-flight mass analyzers, but potentially non-scanning magnetic sector instruments with array detection could provide a viable alternative.

9.2.3.6 Accurate Mass

Accurate mass measurements are generally made using Fourier transform ion cyclotron resonance [1,9,10] or double focusing mass spectrometers of different geometry [1-4] at high-mass resolution. Double focusing geometry employs an electrostatic analyzer to diminish the energy dispersion of the ion source beam and a magnetic sector mass analyzer to disperse the ions according to their mass-to-charge ratio. Generally, a standard for mass calibration is continuously leaked into the ion source and is concurrently present with the sample. Accurate masses are assigned to the calibrant ions and used in an interpolation equation to assign accurate masses for sample ions. Possible empirical compositions and their deviation from the measured mass value for each ion are automatically calculated by software. Either of the above mass spectrometers allow the determination of mass to 10 mDa or better, which is sufficient to determine the exact composition of most low molecular mass ions produced by organic compounds containing common elements. Cost, complexity, sensitivity, and scan rate limit high-resolution mass spectrometers for chromatographic applications. An enduring exception is the detection of dioxins in complex matrices by GC-MS [139-141]. Here, the decrease in sensitivity compared with conventional mass spectrometers is compensated by higher selectivity for the characteristic ions selected for congener quantification. In general, tandem mass spectrometry (section 9.2.4) has established itself as a simpler and more versatile approach to enhance selectivity in chromatographic applications.

Orthogonal acceleration time-of-flight (oaTOF) and hybrid quadrupole orthogonal acceleration time-of-flight mass analyzers (Q-TOF) with a dual sprayer electrospray ion source for addition of a calibration compound, have quickly gained favor for accurate mass measurements of samples separated by liquid chromatography [142-147] and supercritical fluid chromatography [148]. Both analyzer systems are capable of similar mass accuracy, but Q-TOF has the advantages of higher resolution (about 10,000 compared with 4,000) and provides full-scan product ion mass spectra in the MS/MS operating mode. The column effluent and solution of reference compound are introduced separately by orthogonal sprayers into an electrospray ion source. A baffle arrangement, driven by an external stepper motor, allows the solution from each sprayer to be admitted sequentially to the sampling cone region of the mass spectrometer. Each position is indexed to allow storage of the sample and reference data in separate data channels. The reference sprayer is sampled for about 1 s every 5-10 s to maximize sample acquisition time. Subsequent accurate mass measurements are made by averaging and background-subtracting several transient spectra across the chromatographic peak and applying a calibration correction factor obtained from the near simultaneously acquired reference data. The correction factor is calculated from the reference stream, by combining several transient spectra to produce an averaged spectrum at the same time point as the sample data to be mass measured. Accurate mass measurement requires conversion of the data recorded in the continuum mode to

centroid data using software. Accurate composition assignments from less than 10 ng of sample on column have been demonstrated in a number of studies with an uncertainty better than 5 mDa. The Q-TOF is sufficiently versatile, affordable, and user friendly to have quickly found a number of LC-MS applications where identification of trace components in complex mixtures is required [145-147].

9.2.4 Tandem Mass Spectrometry

Tandem mass spectrometers are instruments with a single ion source and two mass analyzers separated by a reaction region [1-3,111,149,150]. They were originally developed for the study of kinetic processes involving energetic ions and their interactions with neutral molecules. These studies contributed to an understanding of the mechanisms of ion fragmentation, gas-phase scales of proton transfer, and other physicochemical properties. General applications in analytical chemistry came much later after the introduction of (usually) simpler systems suitable for structure elucidation and trace level quantification of target compounds in complex matrices. The breakthrough for chromatographic sample inlets was the development of the triple quadrupole (QqQ) tandem mass spectrometer, and more recently, hybrid quadrupole time-of-flight (Q-TOF) instruments. In the intervening years tandem mass spectrometry in time using a single ion trap mass analyzer was developed. All three approaches are in use today.

Electron impact ionization produces stable and reproducible mass spectra containing a rich source of fragment ions suitable for identification purposes. Electron impact ionization is not applicable to non-volatile compounds and the lack of molecular ion information in some mass spectra can be a problem for identifying unknown compounds. Soft ionization techniques (e.g. CI, API, FAB) are applicable to a wider range of compound types than electron impact ionization. They are commonly used with liquid chromatography and capillary electrophoresis interfaces and provide molecular mass information, but relatively few fragment ions for structure elucidation. Collision induced dissociation of mass selected ions with full-scan mass recording of the fragment ions produced allows molecular adduct ions created by soft ionization sources to be used for identification purposes. Collision induced dissociation of mass selected ions with mass selected monitoring of a specific fragment ion is suitable for the trace analysis of target compounds, or specific compound classes, in complex mixtures that require an increase in selectivity for reliable quantification.

The triple quadrupole mass spectrometer consists of a linear combination of three quadrupole mass analyzers, Figure 9.15 [2,56,111]. Only the first and third quadrupole have scan capability. The middle quadrupole, which is sometimes a multipole device of higher order, is a radio frequency-only, gas-filled collision cell with ion focusing properties. All ions above a certain threshold m/z value are transmitted by the middle quadrupole. An offset voltage between the ion source and collision cell can be adjusted to allow the collision energy to be varied between zero and about 200 eV. Inert gases (e.g. He, Ar, Xe) at a pressure of about 2-4 mTorr are commonly used as targets to

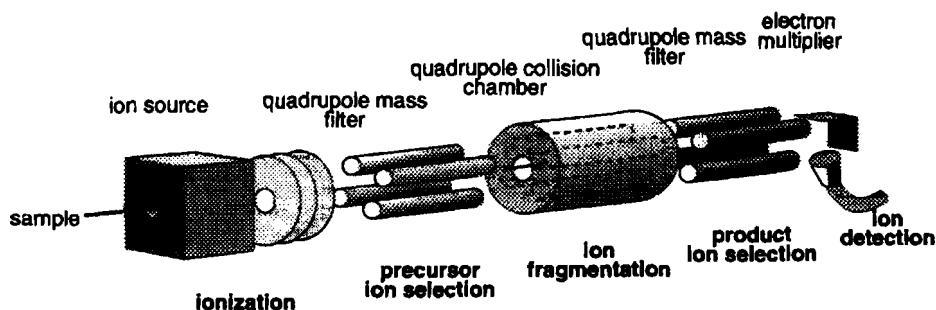


Figure 9.15. Schematic diagram of a triple quadrupole mass spectrometer. (From ref. [150]; ©John Wiley & Sons).

minimize chemical reactions. As a result of collisions, the internal energy of the ion may be increased by conversion of kinetic energy into internal energy. At these relatively low energies the collision duration is long enough to allow direct vibrational excitation [56,57,97,151]. At low collision energies the choice of target gas can have a significant effect on the appearance of the MS/MS mass spectrum.

The triple quadrupole mass spectrometer is operated in four common scan modes summarized in Table 9.5, and of course, it also performs all the functions of a single quadrupole mass analyzer as well [6,56,151-157]. The product-ion scan provides a fingerprint mass spectrum characteristic of the structure of an isolated ion, not unlike an electron-impact mass spectrum. It is used mainly to improve the confidence of analyte identification. A characteristic feature of product-ion scans is the absence of isotope masses. This is because the selected precursor ion contains only the most abundant isotopes. The precursor-ion scan is used to identify the molecular masses of all compounds containing a particular function group or structural fragment representative of a compound class. Precursor-ion scans, for example, are widely used in bioanalysis to identify drug metabolites from the precursor ions giving fragments characteristic of the parent drug. Confirmation of identity is obtained from a product-ion scan. The neutral-loss scan provides a complementary approach to precursor-ion scans for identification of compounds with the same functional group. It allows the selective recognition of all ions that fragment with the loss of a given neutral fragment, for example, the loss of water from alcohols, or carbon dioxide from carboxylic acids. Selected reaction monitoring is used extensively in tandem mass spectrometry for the quantification of target analytes, often at low concentrations, and in complex matrices. For selected reaction monitoring, the first quadrupole is set to transmit a selected precursor ion into the collision cell, and the third quadrupole to monitor in sequence one or more fragment ions derived from the precursor ion. By analyzing only a few selected ions the dwell time for each ion is increased, improving the single-to-noise ratio. Under software control it is easy to program the system to select several groups of precursor and product ion sequences over the course of a chromatographic separation, by multiple reaction monitoring. It is also possible to adjust the collision-offset voltage for each reaction to achieve optimum

Table 9.5

Common scan modes of a triple quadrupole mass spectrometer

(The second quadrupole is operated as a collision cell for collision induced dissociation in all cases)

Scan mode	Description
Product-ion scan	The first quadrupole is set to transmit a selected precursor ion falling within a mass window of usually 1 Da. These ions enter the second quadrupole and are fragmented by collision induced dissociation. The fragment ions are mass resolved by the third quadrupole operated in the full-scan mode. The result is an electron impact-like mass spectrum obtained indirectly from a molecular-adduct ion or other characteristic ion formed in the ion source by a soft ionization method. Also known as a daughter ion scan
Precursor-ion scan	The first quadrupole is set to scan over a selected m/z range. The mass resolved precursor ions sequentially enter the collision cell (second quadrupole) where they are fragmented by collision induced dissociation. The third quadrupole is set to transmit a selected single product ion. The result is a mass spectrum that contains all precursor ions that decompose to produce a common fragment ion. Also known as a parent-ion scan.
Neutral-loss scan	The first and third quadrupoles are linked and scanned at the same speed over the same mass range with a constant mass difference between the two analyzers. Because of the mass offset at any time quadrupole 3 will transmit product ions (if any) with a fixed lower m/z value than the mass selected precursor ions transmitted by quadrupole 1. The result is a mass spectrum containing all the precursor ions that loose a neutral species of selected mass.
Selected-reaction monitoring	Quadrupole 1 is used to select a limited number of precursor ions, which are transmitted sequentially to the collision cell. Quadrupole 3 is set to transmit a limited number of product ions. This technique is used to improve mass detection limits and selectivity for the quantification of target compounds in complex mixtures.

performance. Selected reaction monitoring differs from selected ion monitoring, used with single mass analyzers, in that specific reactants are monitored rather than specific ions, and is subject to less interference from coeluting compounds. Sensitivity is often lower, but this is more than compensated for by the higher specificity.

The hybrid Q-TOF tandem mass spectrometer has the advantage of providing full-scan product ion mass spectra under high-resolution conditions with exact elemental composition assignments for analyte identification [146,158-162]. It is unsuitable for multiple reaction monitoring and neutral-loss scans, since the time-of-flight mass analyzer operates only in the continuous scan mode. The Q-TOF and triple quadrupole tandem mass spectrometer complement each other. The Q-TOF is superior for analyte identification and the triple quadrupole for trace analysis in the multiple reaction monitoring mode on account of its higher sensitivity. The quadrupole mass filter is stepped to sequentially pass ions of increasing mass into the collision cell with a full-scan mass spectra acquired at each step. Automated operation is obtained in the scan-dependent autoswitching mode. A survey scan (single mass analyzer) is performed over a selected mass range and the most abundant ion above a certain threshold value automatically selected as the precursor ion, and the acquisition mode switched from MS to MS/MS operation. The high sensitivity and scan rate of the TOF analyzer

allows product-ion spectra to be generated at several collision energies for each selected precursor ion.

Ion trap mass analyzers select ions in time, providing a straightforward approach to MS^n experiments employing product-ion scans [160,163-170]. Collision induced dissociation occurs within the trap by collision with a gas already present in the trap, or introduced for a short time for this purpose. The collision induced dissociation energy is usually optimized so that about 5-10% of the precursor ion remains at the end of each operation. Ions other than the precursor ion, are ejected from the trap, and the precursor ion fragmented by collision induced dissociation. A new precursor ion of lower mass can be selected from the product ion mass spectrum and the remaining fragment ions ejected from the trap. The sequence can be repeated several times (n times) and is only limited by ion depletion, as long as the collision induced dissociation process yields product ions larger than the lower mass range limit. *In-situ* detection allows true MS^n experiments, in which mass spectra can be obtained after each step of the process. Alternatively, the most-abundant ion can be automatically selected as the precursor ion and fragmented in successive steps. Ion trap product-ion scans are in overall agreement with data obtained by triple quadrupole tandem mass spectrometry. However, the energy available from resonant excitation collisions with helium atoms in the ion trap is significantly lower than that available from collision with argon in a triple quadrupole tandem mass spectrometer. This alters relative intensities of the observed fragments in the product ion mass spectrum.

9.2.5 Data Analysis

A mass spectrometer produces an enormous amount of data, especially in combination with chromatographic sample inlets [4,6,43,48]. The raw data is stored in the form of a three-dimensional array with time, mass-to-charge ratio, and intensity as the independent axes. The data array is generated by repetitively scanning the mass analyzer over a particular mass range during the course of the separation and storing the intensity data for each scan separately. Alternatively, the mass analyzer is set to switch between a few selected ions, and only these ion intensities are stored during the chromatographic separation in selected ion monitoring. Ion trap mass analyzers do not distinguish between full scan and selected ion monitoring, since, in general, all trapped ions are available for analysis. Time-of-flight mass analyzers can provide single ion information, but operate inherently in a continuous scan mode and thus no increase in sensitivity is obtained.

The data system receives information from the detector in the form of an ion current as a function of time. The conversion of the time axis into mass increments is achieved usually through calibration based on the position of known-mass peaks from a standard compound, such as perfluorokerosene or heptacosfluorotributylamine. In addition to time, the Hall voltage for magnetic sector instruments, or the applied voltage for quadrupole instruments, can be used to define the mass scale. A Hall effect probe senses the magnetic field during scanning and provides a time base for mass marking

the spectrum. Whichever method is used, reproducible scan behavior is required, as succeeding spectra are mass converted by interpolation of the calibration data. The mass spectra are usually normalized so that either the most intense peak, or the sum of all mass peaks, is assigned a value of 100%. Other algorithms are available to subtract out background contributions, for example, from column bleed, or to eliminate interference from overlapping chromatographic peaks. The mass spectrometer continues to record data from the separation when no sample peaks are eluting from the column. These scans become the mass spectra of the background. Background scans are recorded as close to the start and shortly after a peak as possible, summed and averaged, and then subtracted from the same number of summed and averaged scans across the chromatographic peak. A number of software approaches, sometimes based on time derivatives or chemometric techniques, are used to subtract interferences from peak overlap or matrix contamination [171-173].

9.2.5.1 Reconstructed Chromatograms

A total-ion current monitor located between the ion source and mass analyzer such that it intercepts a few percent of the total ion beam, provides a continuous record of ion formation in the source. The total-ion current intensity as a function of time, follows the sample flux entering the ion source, and provides a representation of the chromatogram that can be used to identify individual chromatographic peaks. Other approaches allow software reconstruction of the chromatogram from the stored ion intensities. For the continuous scan mode, the data system initiates a scan of the mass analyzer over a fixed mass interval and repeats this process throughout the separation. An advantage of this technique is that a complete record of the separation is available for searching at any time and in different ways, at the expense of disk storage space. In most cases a summary of the total analysis in the form of a total ion chromatogram, or reconstructed chromatogram, is generated. The total ion chromatogram is defined as a normalized plot of the sum of the ion abundance in each mass spectrum, without regard for individual m/z values, as a function of the serially indexed scan number (or time). A scan number containing significant sample information is then easily located from the total ion chromatogram. A base peak chromatogram is obtained by plotting the ion abundance of the base peak in each scan as a function of the scan number (or time). The base peak chromatogram is used to identify sample information in LC-MS, where soft ionization techniques that generate substantial background noise, are commonly used. A mass chromatogram is used to locate specific compounds with known mass spectral properties in a separation. It consists of a plot of the intensity of a single m/z value as a function of the scan number (or time). The specificity is increased by simultaneously displaying the mass chromatograms of several characteristic ions of the substance. The mass chromatograms should maximize in the same scan sequence if the desired substance is present in the sample. The scan number at which this occurs can be identified and the complete mass spectrum obtained, to ensure the compound is correctly identified.

9.2.5.2 Selected Ion Monitoring

It may not be possible to always acquire full-scan mass spectra for identification purposes due to either low sample concentration or matrix interference. However, when the object is to quantify a few substances with known mass spectra, selected ion monitoring (SIM) may provide an acceptable approach. In selected ion monitoring, a few substance-characteristic ions are monitored throughout the separation or a number of different scan windows are programmed, such that the monitored ions change with time, as different target analytes elute from the column. Its principal advantage is improved detection limits, since only the ions of interest are measured at the detector, and the dwell time over which the ion current is recorded for each ion is increased compared to the continuous scan mode. On the other hand, since the full range of ions is not recorded, subsequent examination of the acquired data for additional information is impossible.

Selected ion monitoring lends itself to problems in quantitative trace organic analysis of complex mixtures, and is now a routine tool in many laboratories where additional selectivity at high sensitivity is required, than is provided by standard chromatographic detectors. Selectivity is obtained because mass is a substance-characteristic property. When several masses are recorded sequentially the confidence of the identification is increased, since the probability of two different substances having in common the same retention time and the same characteristic mass ion ratios is small. The selectivity is improved by selecting characteristic ions of high molecular mass, where the chemical background is lower, or by using a high-resolution mass analyzer to isolate the selected ions. Limits of detection (10^{-9} to 10^{-15} g) for selected ion monitoring are at least 10 to 100 times lower than for the full-scan mode. These limits depend on the ionization efficiency of the compound, the fraction of the total ion current carried by the ion monitored, the contribution of background ions to the signal, mass resolution, and the sensitivity of the detector. Quantification is affected by comparing the integrated peak areas or peak heights for the ions of interest with the response obtained from a known amount of standard. Either internal or external standards can be used, but higher accuracy is usually obtained with internal standards. When stable isotope analogs (e.g. ^2H , ^{13}C or ^{18}O) of the substances of interest are used as internal standards, they can be added to the sample at an early stage to correct for losses at all stages of the sample workup. Since they differ from the substance to be determined only by mass, they are ideally suited for selected ion monitoring techniques. In the absence of a stable isotope analog, a homologous compound containing the same m/z ions as the analyte is the next best choice, and may be more readily available or easier to synthesize.

An alternative strategy to selected ion monitoring for increasing the selectivity of an analysis is to monitor ions of a given mass formed by collision induced dissociation from mass selected precursor ions using a tandem mass spectrometer. This process is known as selected reaction monitoring and is described in section 9.2.4.

9.2.5.3 Library Searches

Library search algorithms are commonly provided with mass spectrometer data systems. Their purpose is to assist with the identification of unknown compounds by comparison of their mass spectra to a collection of reference spectra for known compounds [174-176]. Commercial libraries consisting of general organic compounds as well as defined collections of EPA priority pollutants, drugs of abuse, compounds of toxicological relevance, etc., in all up to 370,000 spectra (1999) for about 300,000 compounds, are available. The most general and sophisticated library search procedures are restricted to electron impact mass spectra, since these are reasonably independent of the instrument used for the measurement, and have a higher information content owing to their abundant structure-dependent fragment ions. Most data systems also have software for construction and searching of specialized libraries collected individually.

Two methods are commonly applied for library searches. Identity or retrieval searches assume that the spectrum of the unknown compound is present in the reference library, and only experimental variability prevents a perfect match of the unknown and reference spectra. When no similar spectra are retrieved the only information provided is that the unknown spectrum is not in the library. Similarity or interpretive searches assume that the reference library does not contain a spectrum of the unknown compound, and are designed to produce structural information from which identity might be inferred. Interpretive methods typically employ a predetermined set of spectral features, designed to correlate with the presence of chemical substructures. Searching identifies the library spectra that have features most similar to those of the unknown spectrum. The frequency of occurrence of a substructure in the hit list is used to estimate the probability that it is present in the unknown compound. Two well-developed interpretative search algorithms are SISCOM (Search of Identical and Similar Compounds) and STIRS (the Self-Training Interpretive and Retrieval System) [174-177]. Normally a retrieval search is performed first, and when the results are inconclusive, an interpretive search is implemented. In both cases, success depends on the availability of comprehensive libraries of high-quality reference spectra [178].

The identification of an unknown mass spectrum by retrieval is usually performed in two stages described as screening (or preprocessing) and ranking based on a calculated match factor [174,175,179,180]. Screening seeks to accelerate the library search by abbreviating the mass spectrum while retaining the maximum amount of structural information. Globally significant peaks are used to select a small set of spectra from the reference library most like the unknown. Those methods based on the selection of the N most intense peaks ($N = 3-10$; commonly 8) are easy to implement, but since intense peaks generally represent the most stable and common fragment ions, only a low degree of discrimination is obtained. Giving extra weight to high-mass ions or choosing the two most abundant peaks present every fourteen mass units (Biemann search) improves the discrimination. In a second stage precision matching and ranking are carried out based on the selected library spectra.

An individual mass spectrum can be represented as a single point in hyperspace. The m/z values are the axes and the coordinates are the associated normalized ion

abundance. If two compared spectra are identical, their position in hyperspace will coincide. This is an improbable event, however, if only because of small variations in the measurements. It is more likely that the points will be close in hyperspace, and the distance between them provides an inverse measure of spectral similarity. Spectra that are different will be located as points further apart in hyperspace than spectra that are similar. A large number of algorithms have been described for ranking compared spectra, but the dot product and probability based matching system (PBM) are the most common. The dot product algorithm compares the unknown and reference spectra by computing the cosine of the angle between their vector representations. The probability-based matching system uses the probability that peaks match within an abundance window by chance as weighting factors for mass and abundance along with a variety of rules and correlation tables. In both methods, the results are summarized as a hit list with compounds ranked in descending order of their match factors. When identification is critical a favorable match factor is not enough. The mass spectrum for the reference compound must be run with the same instrument parameters and compared to the unknown. Retention index values for standard separation conditions can be combined with a library search to increase the confidence of identification. However, since certain classes of isomers and homologues produce essentially identical mass spectra, and some types of structural variations produce closely similar mass spectra, mass spectrometry alone cannot be considered an absolute identification method.

When peaks are incompletely separated identification may still be possible using a reverse search. The ability of an algorithm to match two or more components in the mass spectrum of a mixture is aided by requiring only that the peaks of the reference spectrum are present in the unknown spectrum rather than the other way round, as for a normal (or forward) search. The hit list of retrieved library spectra should then represent the compounds in the spectrum of the mixture provided that their spectra are present in the reference library. Subtracting the best-hit library spectrum from the mixture spectrum produces a residual spectrum that can then be matched against the other spectra in the hit list in a forward search. In a sequential process identification of the component spectra may be achieved.

9.2.6 Isotope Ratio Mass Spectrometry

The natural abundance of stable isotopes in organic compounds is not fixed but displays a significant variation reflecting differences in mass discrimination (enantioselectivity and kinetic isotope effects) associated with specific pathways controlling their synthesis and degradation. Stable isotope ratio measurements find numerous applications in geochemistry (fossil biomarkers, formation temperatures, etc.), archaeology (studies of prehistoric diet and lifestyles), authenticity of natural flavors and fragrances (adulteration and counterfeit products) and physiology (metabolism), to name just a few examples. Isotope ratio mass spectrometers provide high precision measurements of isotopic abundance after conversion of the sample into a limited number of gases representative of the original sample (H_2 for $^2\text{H}/^1\text{H}$, CO_2 for $^{13}\text{C}/^{12}\text{C}$ and $^{18}\text{O}/^{16}\text{O}$,

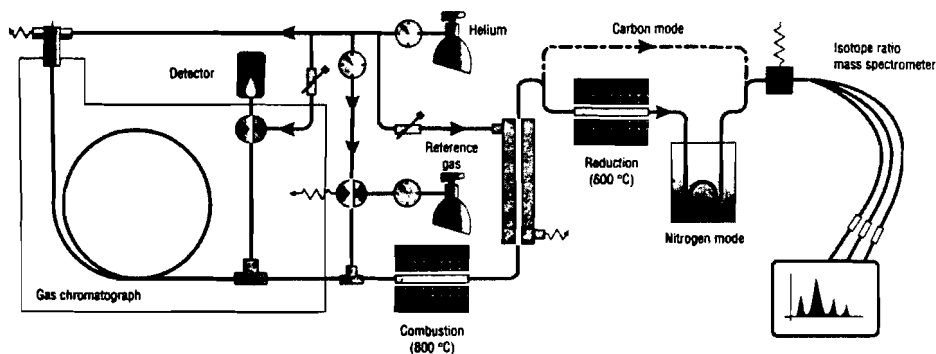


Figure 9.16. Schematic diagram of a GCC-IRMS system for the determination of the relative abundance of stable carbon and nitrogen isotopes. (From ref. [184]; ©Elsevier).

N_2 for $^{15}N/^{14}N$ and SO_2 for $^{34}S/^{32}S$, etc.) Chromatographic applications represent some of the most important applications of isotope ratio mass spectrometry. Gas chromatography-combustion interfaced-isotope ratio mass spectrometry (GCC-IRMS) is used routinely to determine carbon and nitrogen isotope ratios, and to a lesser extent hydrogen and oxygen with greater difficulty [181-185]. Particle beam and moving wire transport interfaces have been used as an interface for liquid chromatography and combustion IRMS, but are not in general use [182].

A typical system for GCC-IRMS is shown in Figure 9.16 [1,2,181,182,184-187]. The column effluent is directed to a combustion furnace and routed through a Nafion or cryogenic water trap before ionization. A fused silica or ceramic tube filled with an oxidized copper or copper-nickel alloy wire loaded with platinum at a temperature above 800°C is used as the combustion reactor. The metal oxide provides oxygen for the reaction and the platinum catalyst assists in driving the reaction to completion. Organic compounds are converted to carbon dioxide, water, nitrogen and nitrogen oxides. The addition of oxygen to the furnace gas during the reaction, or periodically between samples, replenishes the metal oxide surface. Since the metal oxide is the source of oxidant, the combustion interface is unsuitable for the determination of oxygen isotope ratios. Water must be removed from the gas stream for carbon isotope ratio measurements to avoid in-source formation of HCO_2^+ , which interferes with the detection of $^{13}CO_2$ [187,188]. The determination of nitrogen isotope ratios requires addition of a reduction furnace (e.g. a copper wire at 600°C) after the combustion furnace to convert nitrogen oxides to nitrogen and scavenge oxygen released from the combustion furnace [182,186,189]. A cryogenic trap is used to remove carbon dioxide and water. Measurement precision can be improved by placing a short PorapLOT Q capillary column between the water trap and ion source to separate traces of nitric oxide from nitrogen, which react in the ion source. Oxygen isotopes have been determined using a pyrolytic interface to convert oxygen-containing organic compounds to carbon monoxide for IRMS measurements [190]. Hydrogen and oxygen isotopes have been

determined after pyrolytic conversion of organic compounds to hydrogen and carbon monoxide over a nickelized carbon packing at temperatures above 1000°C [191]. Inadequate separation of HD and helium ions by the mass spectrometer can be solved by using a palladium foil filter to selectively isolate hydrogen isotopes, or by redesign of the mass spectrometer [192].

An open split interface is commonly used to maintain a constant flow of gas into the ion source. The ion source is usually an electron impact source with a dual inlet design, operated at relatively high pressures, to maximize ion formation. A correction method for hydrogen isotope measurements to account for the formation of H_3^+ by hydrogen ion-hydrogen molecule collisions is required [182,193]. A dual-inlet source is employed to facilitate addition of a reference gas diluted in helium for calibration. The reference gas is automatically admitted to the ion source through a separate inlet during empty regions in the chromatogram, or before and after a separated peak. Alternatively, a reference standard of known isotopic composition can be included in the chromatogram for calibration. Since isotope ratio measurements are not absolute measurements, a reference standard is always required for high precision measurements. The mass analyzer is always a magnetic sector with a resolution of about 100 and operated in the non-scan mode. These conditions are selected to optimize stability and ion transmission, which are the two most important features of an isotope ratio mass spectrometer. Faraday cups, one for each ion beam of interest, are used for detection. Simultaneous detection improves precision by minimizing the effects of fluctuations in the ion beam intensity.

Isotope ratio mass spectrometry provides information for the isotopic abundance of an analyte gas relative to the known isotope ratio of a reference compound. Primary standards are available for calibration and to provide traceability to facilitate comparison of results from different laboratories. The peak area for each isomer is integrated and the corresponding ratios calculated. Since the small variations of the heavier isotopes are of the order of 0.001 to 0.05 atom percent, the δ -notation in units of parts per thousand ($‰$) is used to report changes in isotope abundance, where $\delta_s = 1000 (R_s/R_{\text{std}} - 1)$, and R_s is the measured isotope ratio for the sample and R_{std} the isotope ratio for the reference standard [2,182,194,195]. Minimum sample quantities for isotope ratio measurements vary tremendously with the operating conditions and other factors, but typically carbon isotope ratios can be determined for samples containing about 10 ng of carbon.

9.3 FOURIER TRANSFORM INFRARED SPECTROMETRY

Absorption in the infrared region of the spectrum by organic compounds arises as a result of bending and stretching of covalent bonds at different characteristic frequencies. The frequency of vibration is related to both bond order and the mass of the atom attached to the bond. Many organic compounds have a large number of relatively narrow absorption bands in the mid-infrared region ($1600\text{--}900\text{ cm}^{-1}$). These

absorptions are highly specific and provide detailed structural information. Infrared spectra are particularly useful for the identification of functional groups and can usually distinguish between positional and conformational isomers. Except by comparison with an authentic spectrum, however, it is rarely possible to identify an unknown compound from its infrared spectrum alone. Infrared spectrometry is particularly poor at distinguishing between homologues without additional molecular mass information. Since this information is readily available from mass spectrometry, the two techniques complement each other. Knowledge of a compound's infrared spectrum aids in the interpretation of fragmentation pathways in the mass spectrum, and fully integrated GC-FTIR-MS instruments are available (section 9.3.2).

Dispersive (grating or prism) infrared spectrometers are relatively slow, insensitive and unsuitable for recording spectral information on the fly from rapidly eluting chromatographic peaks. The introduction of interferometric recording instruments and the improved sensitivity of the mercury-cadmium-telluride photoconductivity detector, are credited with making possible the development of coupled chromatographic and infrared spectrometric instruments [2,196-200]. The infrared spectrum is recorded by a Michelson interferometer consisting of two mirrors at right angles to one another and a beam splitter bisecting the angle between them. One of the mirrors is stationary while the other is moved at a constant velocity. Light from the source is split into two out-of-phase beams, which are recombined at the beam splitter, and for any particular wavelength constructively or destructively interfere, depending on the difference in optical paths between the two branches of the interferometer. For a broadband emission source, each frequency comes in and out of phase at a characteristic optical path difference, and the superposition of all frequencies produces the observed interferogram. The combined beam then traverses the sample compartment and is focused on the detector. The detector signal is sampled at precise intervals during the mirror scan. Both the sampling frequency and mirror velocity are synchronized by modulation of an auxiliary reference beam from a helium-neon laser. Fourier transform of the detector signal provides a single beam spectrum, which may then be ratioed against a background spectrum to provide the customary transmittance (or absorbance) versus wavenumber infrared spectrum. Full-scan infrared spectra can be recorded at a rate of 1-20 per second (depending on resolution), and individual scans summed during the elution of a chromatographic peak for increased sensitivity

9.3.1 Chromatographic Interfaces

Chromatographic interfaces are based on three common approaches; the flow-through cell (light pipe) and solvent elimination with either matrix isolation or cold trapping [2,198,201]. Flow-through cells provide a simple and convenient interface for GC-FTIR, since typical mobile phases are transparent in the mid-infrared region. Mobile phase elimination interfaces are used primarily to increase sensitivity, and to obtain high-resolution or condensed phase spectra, for improved confidence of identification by library search techniques. Vapor phase spectra have characteristic broad absorption

bands and available infrared spectral libraries are smaller than for condensed phase spectra. Liquids and supercritical fluids have strong absorption bands in the mid-infrared region making flow cells unacceptable for some applications. Common mobile phases may completely obscure sample bands in some spectral regions. Spectral subtraction of solvent bands is almost impossible when the composition of the mobile phase is changing, for example, during gradient elution. The flow cell approach is thus less attractive for liquid and supercritical fluid chromatography, and in general, mobile phase elimination interfaces are preferred.

9.3.1.1 Gas Chromatography-Fourier Transform Infrared Spectrometry

The simplest interface for GC-FTIR is a flow-through cell, light pipe, connected to the gas chromatograph by a length of heated fused-silica capillary tube [2,196,198,202-208]. The light pipe consists of a heated glass tube, internally coated with a layer of gold, and closed at either end with infrared transparent windows. The chromatographic effluent enters at the side close to one window and exits from the side close to the opposite window. The purpose of the gold layer is to increase the optical path length by multiple internal reflections of the light beam. For optimum performance the volume of the light pipe should be no larger than the peak volume determined by the full-width at half-height of the main peaks in the chromatogram. Makeup gas added to the column effluent can be used to preserve resolution at the expense of sensitivity. If the volume of the light pipe significantly exceeds the peak volume, as determined by its width at half-height, resolution may be compromised by failure to sweep out the tail of one peak before the next peak enters the light pipe. Since it is inconvenient to change light pipes to match each chromatogram, it is general practice to select certain light pipe volumes, and then set the chromatographic conditions so that the half-height peak volumes of each peak (or as many as possible) are equal to, or slightly greater than, the light pipe volume. For open tubular columns, light pipes about 10 cm long with an internal diameter of 1 mm with volumes of 100-150 μl , are commonly used. For packed columns, more typical dimensions are lengths from 20 to 100 cm and internal diameters from 1 to 3 mm with volumes of 0.8 to 5 ml. Full spectrum recording of identifiable spectra requires about 10-25 ng per component for strong absorbers and about 100 ng for weak absorbers. Vapor phase spectra contain relatively broad bands and a resolution of 8 cm^{-1} is typically used to record spectra. The popularity of the light pipe interface is a result of its simple design and possibility for unattended operation. The principal problems are low sensitivity, lower transmission of infrared energy by the light pipe at higher temperatures, and dead volume restrictions for fast and high-resolution separations. The direct deposit interfaces can be seen as an attempt to overcome the sensitivity limitation, and to provide higher spectral resolution for the identification of compounds with similar infrared spectra. This is achieved by immobilizing the separated peaks into a small volume interrogated by a condensed beam and suitable optics, and by using signal-averaging techniques.

The interface for matrix isolation consists of a high vacuum chamber housing a motorized gold-plated collection disk and a helium refrigeration unit, Figure 9.17

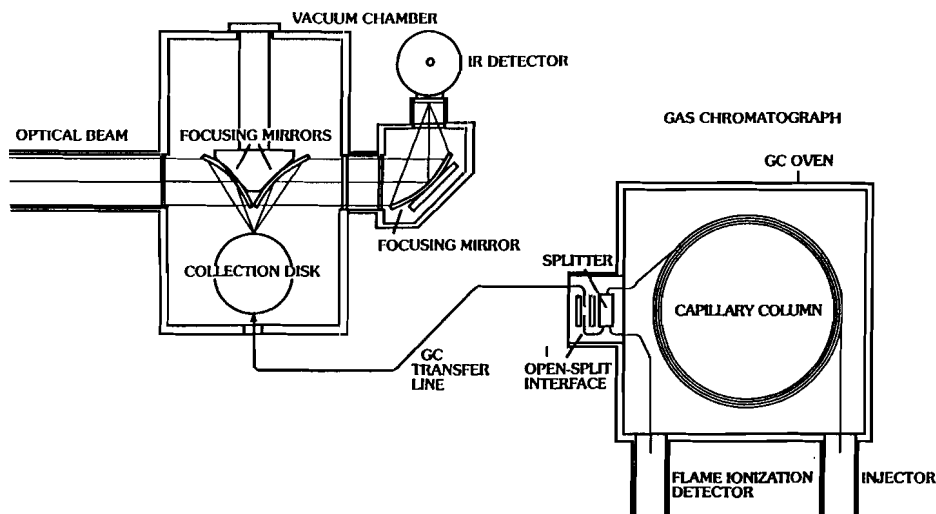


Figure 9.17. Schematic diagram of a GC-FTIR interface based on matrix isolation. (From ref. [193]; ©Marcel Dekker).

[197,198,202,203,207-214]. Helium containing argon ($\approx 1\%$ v/v) is used as carrier gas, or a helium-argon gas mixture is added postcolumn to the column effluent. Usually the column effluent is split between a flame ionization detector and open split interface, similar in design to GC-MS interfaces (section 9.2.2.1). The function of the open split interface is to provide a constant flow of gas to the vacuum chamber, and to decouple the operation of the gas chromatograph from the cryogenic matrix isolation unit. This facilitates configuration changes to the gas chromatograph without effecting the operation of the vacuum chamber, reducing downtime while it is pumped out and decontaminated. The open split coupling also allows diversion of solvent peaks and other interfering components from the cryogenic matrix isolation apparatus. A heated fused-silica capillary transfer line (1 m x 0.18 mm I.D.) connects the open split interface to the nozzle that deposits the sample and matrix as a solid trace about 300 μm wide on the moving collection disk maintained at about 10 K by the helium refrigeration unit. The rotation of the collection disk is correlated to the retention times determined by an auxiliary detector (FID, MS, etc.) by the data system. The collection disk can store up to about 5 h of chromatograms frozen at one time. Under the prevailing conditions for deposition, the helium remains gaseous and is pumped away, while the sample components are trapped in a matrix of solid argon. The strip of argon is subsequently rotated through the infrared beam and a series of absorption spectra are measured by reflectance. The critical parameters for optimizing the interface are: the deposition rate; the ratio of sample to matrix (which should be $< 1\%$ sample); temperature of the collection disk; the distance between the tip of the transfer capillary at the nozzle and the

collection disk; and the vacuum chamber pressure. To obtain low identification limits, the sample spot size and infrared beam dimensions have to be carefully matched.

In the matrix of solid argon, intermolecular interactions among sample molecules and with the matrix are minimized. The lack of significant intermolecular interactions and the absence of molecular rotation lead to infrared spectra having narrow lines and detailed fine structure typically recorded at a resolution of 1 cm^{-1} . Since the separation and measurement process are separated in time, signal averaging techniques can be applied to improve sensitivity compared with light pipe measurements. Full spectra can typically be recorded from about 0.01-0.10 ng of strong absorbers and 0.20-1.0 ng of average absorbers. The high cost and complex nature of the apparatus, the fact that data is not provided in real time, and the need for special matrix isolation libraries for computer searching, have restricted the general use of the matrix isolation interface to specialized research laboratories.

The direct deposit interface provides a similar and somewhat simpler device to the matrix isolation interface, for isolating sample components from the carrier gas [198,199,206,208,214-219]. The gas chromatograph can be connected directly to the deposition interface by a heated fused-silica capillary tube, but a connection through an open split interface provides more versatility, as discussed for the matrix isolation interface. The analytes are deposited on a moving zinc selenide window, housed in a vacuum chamber. A heated fused silica tip of 50-150 μm internal diameter is fixed at the end of the transfer line and located about 30 μm above the surface of the zinc selenide window, which is cooled to 80 K with liquid nitrogen. The window is continually shifted during deposition by means of a stepper motor drive, and the deposited sample spots of 100-150 μm diameter, are passed through the beam of an FTIR microscope a few seconds after deposition. Microscope optics are used to match the FTIR beam diameter to the sample spot size. Spectra are typically recorded at a resolution of 8 cm^{-1} and in all but minor details are usually identical to solid phase spectra measured in potassium bromide disks. This approach has the advantage of providing near real-time chromatogram reconstruction and spectra, minimum identification quantities similar to matrix isolation spectra, and provides spectra that can be identified by searching solid phase infrared libraries. The most serious problem is ingress of water (and to a lesser extent carbon dioxide) deposited as ice on the zinc selenide window that interferes in the recording of the sample spectra. Sample spots can be stored in the frozen state on the zinc selenide window, and their infrared spectra measured later, using signal-averaging techniques to improve sensitivity.

9.3.1.2 Liquid Chromatography-Fourier Transform Infrared Spectrometry

Most common solvents used in liquid chromatography show significant absorption in the mid-infrared region, and consequently, solvent elimination interfaces are required for many applications. Flow cell interfaces are generally restricted to monitoring selected frequency ranges for functional group detection. Typical flow cells are similar to UV detector cells with infrared transparent windows [198,220-222]. Short path length cells ensure sufficient energy reaches the sensor. Path lengths less than 0.2 mm are

used for organic solvents and less than 0.03 mm for water-organic solvent mixtures. Consequently, sensitivity is low, and sample amounts in the (high) microgram range are required to give useful spectral information. In those regions of the spectrum where the mobile phase shows appreciable absorption, sample information may be completely lost. Solvent subtraction is often impossible for gradient separations. Current applications are largely limited to size-exclusion chromatography, where high sample concentrations and isocratic separations are common and favorable solvents for infrared measurements, such as chloroalkanes, are suitable for the separation.

Solvent elimination interfaces offer three primary advantages over the flow cell approach. Firstly, interference-free spectra of the separated compounds can be obtained. Secondly, the mobile phase composition and elution method can be freely selected, virtually independent of spectrometer requirements. Finally, since each chromatogram is stored in the interface, spectra can be recorded free of time constraints, allowing sensitivity to be enhanced by signal averaging. The common solvents used in liquid chromatography, however, are not as easily removed as gases. Consequently, solvent elimination interfaces are more complex and difficult to use than those described for gas chromatography (section 9.3.1.2) [198,222-227]. As well as the solvent elimination interfaces developed specifically for LC-FTIR, LC-MS interfaces (electrospray, thermospray and particle beam) have been used for LC-FTIR without being widely adopted [222,225,228]. Since in all these interfaces solvent elimination is achieved by evaporation, the sample must be less volatile than the mobile phase.

Solvent elimination is achieved by spraying the chromatographic eluent through a focusing nozzle and depositing the separated sample components on the surface of a moving infrared transparent window or reflective metallic mirror. Either a pneumatic nebulizer or ultrasonic nebulizers, is used to convert the column eluent into fine droplets, which are stripped of solvent as they move to the collector surface. The tip of the spray capillary of the pneumatic nebulizer is surrounded by a flowing stream of hot nitrogen gas, which provides sufficient energy to evaporate the mobile phase, and to contain the spray in a tight, focused cone at the surface of the collection disk. Acceptable flow rates for pneumatic nebulization are limited to less than 1.0 ml/min for totally organic mobile phases and less than 0.3 ml/min for aqueous mobile phases. Alternatively, the column eluent and gas flow are combined at an ultrasonic nebulizer to form a fine spray, which is then desolvated at low pressure in a heated transfer tube that directs the sample particles to the surface of a moving zinc selenide window. To match the acceptance flow rates of the pneumatic nebulizers to the column flow rate; postcolumn splitting of the column eluent is frequently used. Buffers and other nonvolatile mobile phase components can interfere in the measurement of sample spectra.

There are two alternatives for acquiring infrared spectra from the deposited samples. Samples deposited on an optically flat germanium disk with an aluminum backing are transferred to a separate module for reflectance-absorption measurements. The disk is rotated continuously during sample collection, with the result that the chromatogram is deposited as a track near the perimeter of the disk. Rotation of the disk in the spectrometer module allows either a continuous scan of the deposited chromatogram

or static scans at selected locations. Infrared energy is focused to a narrow beam and directed through the sample. The beam passes through the germanium, reflects off the aluminium coating on the lower surface, and is retransmitted through the sample, collimated, and directed to the detector. The infrared spectra of sample residues deposited onto a moving zinc selenide window are measured by transmission shortly after deposition in a vacuum chamber containing the deposition system and optical components for the infrared beam. The sample can also be stored on the zinc selenide window for measurement at a later time. The transmission interface is generally the most sensitive, with full spectra obtained from spots containing about 1-10 ng of sample. Reflectance-absorption measurements usually require about 2-5 fold more sample. Owing to diffuse spray characteristics and poor sample transfer minimum identifiable quantities vary widely for different conditions, but usually fall in the range of nanograms to micrograms for typical samples. Spectral quality is also influenced by the deposited state of the sample. Samples deposited in an amorphous form often exhibit broadened absorption bands and may differ from spectra for the crystalline form present in reference libraries.

9.3.1.3 Supercritical Fluid Chromatography-Fourier Transform Infrared Spectrometry

Infrared spectra are measured in SFC-FTIR using either a pressure-stable, thermostatted flow cell or solvent elimination interface with sample deposition on a moving infrared transparent zinc selenide window [198,229-224]. The practice of supercritical fluid chromatography is dominated by the use of carbon dioxide as the main mobile phase with open tubular columns and carbon dioxide with various amounts of polar organic solvents for packed columns. Since most mobile phase components are gases or volatile liquids at room temperature and pressure elimination of the mobile phase occurs under more favorable conditions than for liquid chromatography.

Carbon dioxide has many favorable infrared spectral properties when compared with the solvents commonly used in liquid chromatography. Only the absorption region between 3475 to 3850 cm^{-1} and 2040 to 2575 cm^{-1} are completely inaccessible due to strong absorption by carbon dioxide. Information may be lost or reduced in the region between 1200 and 1475 cm^{-1} due to absorption resulting from Fermi resonance, the magnitude of which depends on the carbon dioxide density. For short-path length flow cells, the background may be effectively subtracted from the spectrum. FTIR spectra recorded in supercritical fluid carbon dioxide differ from those recorded in the vapor or condensed phase due to shifts of the maximum absorbances, variation of bandwidths, and modification of the intensity distribution. Stretching vibrations are more sensitive to carbon dioxide density variation than bending vibrations; furthermore wavenumber shifts are relatively modest for weak polar functional groups ($< 2\text{ cm}^{-1}$), but more important for polar functional groups (up to 10 cm^{-1}). Direct infrared measurements may be impossible for mixtures of carbon dioxide with organic solvents due to the difficulty of subtracting the background contribution from the modifier. Besides carbon dioxide, supercritical fluid xenon has been proposed as a solvent for infrared spectroscopy due to its favorable critical constants and transparency throughout the

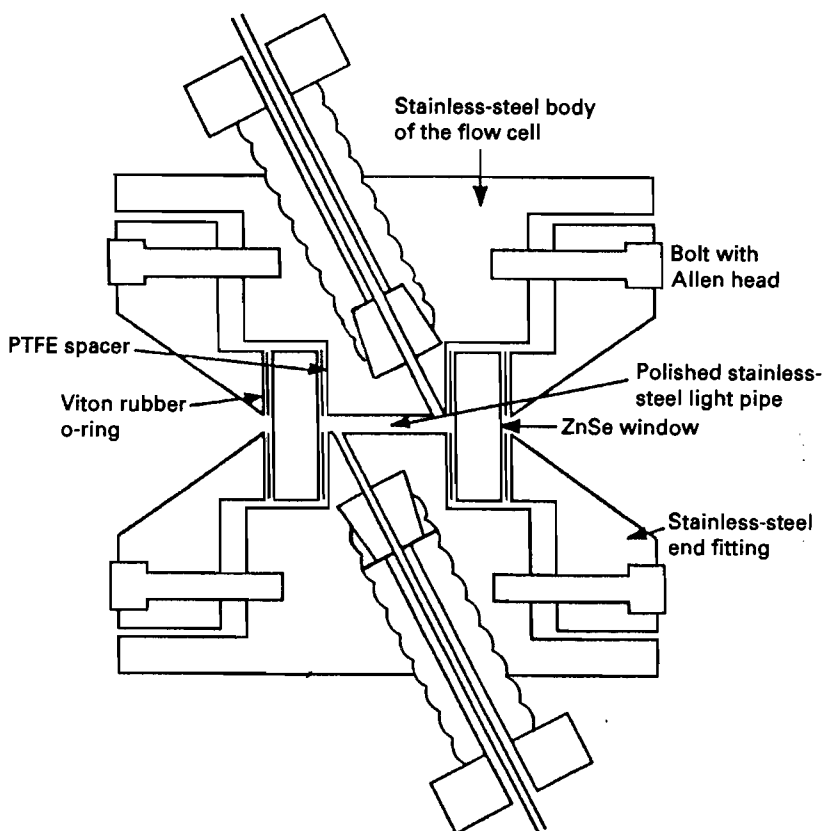


Figure 9.18. Schematic diagram of a high-pressure flow cell for SFC-FTIR.

mid-infrared region [235]. High cost and limited solubilizing properties for polar solutes have limited its use so far.

Typical flow cells are cylindrical in shape and polished internally to maximize reflection with infrared transparent end-windows made from calcium fluoride or zinc selenide, Figure 9.18 [229,231,236]. The flow cell is positioned between the column and restrictor or backpressure regulator, and must be capable of withstanding the pressure and temperature required to optimize the separation conditions. Typical cell volumes vary from 0.8 to 8.0 μl representing a compromise between maintaining chromatographic resolution and providing reasonable sample mass detectability. The cell diameter is dictated by the optics of the spectrometer, and cannot be easily reduced below about 0.5 mm without a significant increase in noise. Similarly, the cell path length cannot be increased beyond 5 mm due to excessive absorption of the infrared radiation by the mobile phase (carbon dioxide). Path lengths for flow cells used with open tubular columns are limited by the contribution of the cell volume to extracolumn

band broadening, and are typically 1-5 mm (cell volumes $> 0.8 \mu\text{l}$). These cell volumes are still larger than desirable, and even if makeup flow is added prior to the cell to reduce dead volumes (and also sensitivity), some loss in chromatographic resolution must be accepted. Sample amounts of at least 10-100 ng are required for structure identification.

Elimination of the mobile phase before infrared measurements allows the spectrum to be obtained without interference from mobile phase absorption bands. It also eliminates the influence of temperature and pressure on the position and shape of weak absorption bands, thereby allowing spectral searching against standard condensed phase libraries for identification. The chromatogram is deposited in a vacuum chamber on a moving zinc selenide window from a heated restrictor located about $75 \mu\text{m}$ above the window and connected to the column by a heated capillary transfer line [203,229,232,233]. The zinc selenide window is cooled to about -10°C to trap volatile sample components without condensing the mobile phase. The window may be held at a higher temperature for samples of low volatility. Liquid samples may not solidify rapidly enough at modest temperatures and may be blown off or spread over the smooth window surface by the high velocity gaseous mobile phase created at the restrictor tip. The optimum speed to translate the window for sample disposition is such that it moves a distance equal to the width of the track in a time equal to the full-width at half-height of the narrowest peak in the chromatogram. This provides a reasonable compromise between retaining the chromatographic resolution and maximizing the thickness of the deposited sample for higher sensitivity. Subsequently, the sample is transported through the beam of an infrared microscope shortly after deposition, or the chromatogram is interrogated at a later time using signal-averaging techniques to improve sensitivity. Identifiable spectra can be obtained from samples of about 0.5 to 10 ng.

9.3.1.4 Thin-Layer Chromatography-Fourier Transform Infrared Spectrometry and Thin-Layer Chromatography-Raman Spectrometry

Infrared spectra of sample zones on a thin-layer plate can be obtained by either *in situ* measurements using a diffuse reflectance spectrometer or by transmission measurements after transfer of the sample zones to an infrared transparent substrate [96,198,237-240]. The layers typically used for separations have strong infrared absorption between $3700\text{-}3100$ and $1650\text{-}800 \text{ cm}^{-1}$ for silica (and bonded phases) and $4000\text{-}3500$ and $2700\text{-}1500 \text{ cm}^{-1}$ for cellulose preventing useful sample information from being obtained in these regions. Both infrared reconstructed chromatograms and individual spectra can be obtained with a specially designed gas purged DRIFT unit connected to an FTIR spectrometer. The special arrangement of mirrors largely eliminates the specular reflectance from the layer surface and allows the diffuse reflectance component, which contains the sample absorption information, to be collected and focused at the detector. In the usual arrangement the position of the infrared beam is fixed and the layer is moved through it on a motorized x-y translation stage. To acquire useful spectra the background infrared absorption of the layer has to be subtracted from the spectra of the sample zones. This is usually accomplished by measuring a representative blank plate spectrum that matches the background of the

sample zones as closely as possible. Normally about 100 ng of material is required to detect individual functional groups and about 1 μ g for partial measurement of a spectrum using signal-averaging techniques. Strong interactions between the sample and the layer cause shifts in absorption bands in the DRIFT infrared spectra compared with spectra obtained from potassium bromide disks. Library searches usually require reference spectra measured under similar conditions to the DRIFT measurements. For compounds with a chromophore, UV detection usually provides lower detection limits and a more straightforward approach to quantification [240].

Transferring the TLC chromatogram without significant loss of chromatographic resolution to an infrared transparent substrate can circumvent the limitations of *in situ* measurements [96,198,241]. Numerous methods have been described for this purpose, most are manual and time consuming, some allow evaluation of a few selected sample zones only. In one general approach the transfer is achieved by eluting the sample with a strong solvent in the direction orthogonal to the direction of development, and towards a metal strip placed along one edge of the layer. The strip contains a series of sample cups filled with a suitable powdered infrared substrate and a wick to aid sample transfer. A controlled flow of air over the surface of the powder evaporates the solvent, leaving the sample components deposited on the surface. The metal strip is placed in an automated diffuse reflectance sample chamber for infrared measurements. In this way, the amount of sample required for identification can be reduced by an order of magnitude and the loss of information due to absorption by the sorbent eliminated.

Although thin-layer adsorbents, such as silica, have weak Raman absorption bands, *in situ* Raman spectra of adsorbed compounds are generally weak resulting in poor sensitivity. Significant improvements in sensitivity are possible, however, using resonance Raman and/or surface enhanced Raman spectroscopy [96,242-245]. Surface enhanced Raman spectra can be observed for compounds adsorbed on rough metallic surfaces, usually silver or gold, while resonance Raman spectra are obtained when the frequency of the exciting radiation coincides with an electronic absorption band of the sample. The silver colloid for surface enhanced Raman spectroscopy is applied to the layer by a light spraying after development or by overspotting individual sample zones. Spectra suitable for identification can be obtained from about 2-10 ng of some samples, but surface enhanced Raman is not a universal technique, since it relies on adsorption to the silver sol, a property that is strongly sample dependent. Surface enhanced Raman spectroscopy is inherently an identification technique, as the Raman emission intensity is variable from different sample zones. Compared with normal Raman spectra, surface enhanced Raman spectra often show small changes in relative intensity, band position, and band shape.

9.3.2 Coupled Interfaces with Mass Spectrometry

Infrared and mass spectrometers provide complementary information highly desirable for identification of unknown compounds. Mass spectral libraries are significantly larger than those available for infrared spectra, so the main function of infrared information

is to confirm an identification by providing functional group and isomer-specific information. The characteristic feature of coupled systems is the integrated and near simultaneous collection and analysis of the spectral data. Systems for gas chromatography coupled to infrared and mass spectrometry (GC-FTIR-MS) have been available for a number of years. By comparison, coupled systems for liquid chromatography have been used to a limited extent, and usually not with conjoint data analysis.

Coupling of infrared and mass spectrometers to a gas chromatograph has been achieved by either splitting the column effluent between the spectrometers in a parallel configuration (this is also the general approach for liquid chromatography), or in a serial configuration by passing the column effluent through a flow cell (light pipe) and then to the mass spectrometer [204,206,216,246-248]. In the parallel configuration the carrier gas flow (and makeup gas flow if used) is split between the two instruments, such that about 90-99% of the flow goes to the infrared spectrometer. In this way, the different sensitivity of the two instruments can be matched, and optimum flow provided to each spectrometer. For serial coupling, makeup gas is added to the column effluent to match the requirements of the light pipe for optimum sensitivity, and the exit flow is diverted to the mass spectrometer using a splitter and jet separator or open split coupling to reduce the gas flow into the mass spectrometer to its optimum value. The open split interface is the most versatile, since it is easy to optimize for different flow rates, and can function as a splitter when high flow rates are used in the interface to minimize resolution loss and to maximize sensitivity of the light pipe. The low sensitivity of the light pipe interface compared to the sensitivity of mass spectrometry remains the primary limitation for problem selection, since the compromises made in the chromatography are done to accommodate the circumstances of infrared detection, and are unrelated to the capabilities of the mass spectrometer. The matrix isolation and direct deposition interfaces are superior in terms of matching the sensitivity of the infrared spectrometer to that of the mass spectrometer, but requires a parallel coupling.

9.3.3 Data Analysis

Computer acquisition provides attendant advantages, such as real time chromatogram plots, background subtraction, peak deconvolution, and library search facilities, as well as calculation and storage of the infrared spectra [2,171,196-199]. Interferograms are measured continuously throughout the separation, at a rate that depends on the selected spectral resolution, typically between 1 to 20 interferograms per second. It is common practice to average blocks of interferograms for a period of time that is slightly less than the full-width at half-height of the narrowest peak in the chromatogram. The single beam spectrum is then computed from the signal-averaged interferogram and ratioed against an appropriate background spectrum. The resulting transmittance spectrum is converted to a linear absorbance format for display and use in library searches. Storage interfaces, such as sample deposition interfaces, allow measurement of spectra free of time constraints, and the number of averaged spectra can be optimized to obtain a desired signal-to-noise ratio and identifiable spectra obtained from smaller sample sizes.

9.3.3.1 Reconstructed Chromatograms

Chromatograms can be reconstructed from stored interferograms using either the Gram-Schmidt vector orthogonalization algorithm or by integrating the absorbance between specified wavenumber limits. The latter technique yields a chromatogram that is characteristic of only those functional groups that give rise to an absorption band in the specified spectral region. The reconstructed chromatograms are used to select interferograms containing useful spectral information for more detailed examination, and to provide quantitative information for sample components.

The Gram-Schmidt orthogonalization method is a normalization routine employing interferometric data without prior Fourier transformation. It is the preferred method for chromatogram reconstruction using a wide frequency window because it is computationally simpler, and provides greater sensitivity than integrated absorbance methods. A reference set of interferogram segments is collected that contain no sample information, and used to construct a set of orthogonal basis vectors in multidimensional hyperspace. For chromatogram reconstruction, each interferogram collected following the start of the separation is used to compute a resultant vector orthogonal to the basis vectors. The magnitude of the difference between the computed vectors and the basis vectors is approximately proportional to the sample amount. The Gram-Schmidt reconstructed chromatogram is non-selective, but some compounds are weaker infrared absorbers than others, which is reflected in the peak areas of the chromatogram. The slope of the calibration curves from the Gram-Schmidt plots is influenced by changes in background contributions from contaminants, particularly water vapor, requiring that the carrier gas and spectrometer purge gas are rigorously dried.

An alternative to the Gram-Schmidt method is to construct a chromatogram from either the integrated or maximum absorbance of the spectrum obtained from the Fourier transform of the interferograms. Both methods are generally used to create selective chromatograms by integrating over a narrow frequency range, characteristic of a particular functional group. The integrated absorbance method calculates the total absorbance in a selected frequency range of each transformed spectrum. The maximum absorbance method calculates the maximum absorbance value within the selected frequency range of the transformed interferogram, and uses it as an ordinate value corresponding to a retention time in the reconstruction. The maximum absorbance method is more sensitive than the integrated absorbance method, and does not decline if the frequency window is much larger than the bandwidth of the peak within the window. The peak areas in the chromatogram are proportional to concentration, and therefore, can be utilized for quantification after calibration.

9.3.3.2 Library Searches

There are two general approaches for computer-aided identification of infrared spectra of unknown compounds [173,196-199,248-250]. The most common approach uses software designed to identify an unknown spectrum by its similarity to a limited number of reference spectra selected from a general or customized library of reference spectra measured under similar conditions (e.g. vapor phase, solid phase, etc.) Commercial

infrared libraries fall into two groups. Relatively small comprehensive libraries for a group of compounds defined by specific applications, and larger general libraries of up to about 190,000 compounds (1998). Solid-phase libraries are the largest with vapor phase and matrix isolation libraries considerably smaller. The second, and less common approach, uses artificial intelligence and chemometrics software for structural interpretation, based on the assignment of prominent absorption bands to possible structural features of the unknown compound.

For computer matching the unknown and reference spectra are first scaled so that the most intense band for each spectrum has the same absorbance (usually 1). The unknown spectrum and the reference spectra are then represented as points in multidimensional space, each dimension corresponding to a single wavenumber in the spectrum. The Euclidean distance between the unknown and each reference spectrum in multidimensional space is calculated and the distance converted to a hit quality index (HQI). The index is usually scaled such that values close to zero or some ordinal value (e.g. 1000) indicate a high level of similarity for the compared spectra. However, unequivocal identification cannot be made based on an optimized score alone. A side-by-side visual comparison of the unknown spectrum and reference spectra of the top few hits is recommended to confirm identifications. In addition, software is available for the identification of unknown compounds in a mixture [248]. For example, principal component regression can be used to find the greatest similarity between the spectrum of the mixture and a library spectrum. For subsequent identification of the remaining components, all the similarities of the target compound are removed from the mixture spectrum, and the residual spectrum searched against the library spectra again. This procedure is repeated until the similarity index of the residual mixture spectrum against the library spectra falls below a preset threshold. Identification is possible by this method if the unknown spectra are present in the reference library.

9.4 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Nuclear magnetic resonance (NMR) detects signals from the absorption of radio frequency energy by nuclei placed in a strong and homogeneous magnetic field. The circulation of electrons around the nucleus creates local magnetic environments, resulting in characteristic chemical shifts suitable for functional group identification. The presence of coupling between pairs of nuclei extending over 1 to 3 bonds establishes the connectivity between atoms and provides a powerful tool for structure determination including stereochemical information. Thus, the particular strengths of NMR are its ability to differentiate between most structural, conformational, and optical isomers, and the possibility of complete structural elucidation of unknown compounds with a known molecular mass. The combination of mass spectrometry and NMR spectroscopy has long been considered the primary tools for structure elucidation in organic chemistry. Their conjoint application to chemical analysis has only been limited in the past by the relatively poor sensitivity of NMR spectroscopy compared with mass spectrometry.

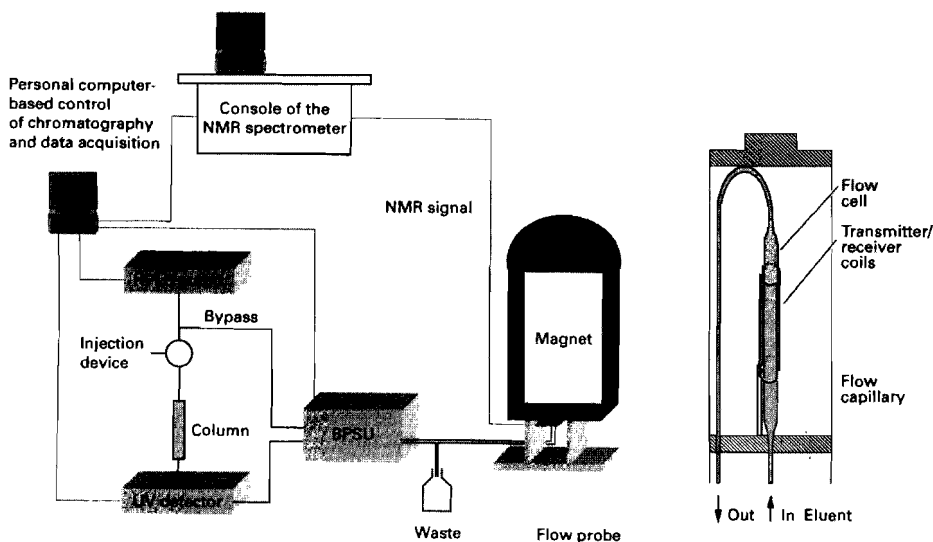


Figure 9.19. Typical system for LC-NMR (BPSU = Bruker peak sampling unit) and expanded view of an NMR flow cell. (From ref. [265]; ©Academic Press)

The general coupling of chromatographic techniques to NMR spectroscopy was slow to develop. Realization of practically useful coupled systems faced serious difficulties related to sample size requirements, time constraints and dynamic range of NMR spectrometers, and the large dead volume of interfaces. The resolution of these problems has benefited from the continuous improvements in flow probe interface designs, high field magnets, digital electronics, and software, which is an on-going process without signs of having reached maturity. Only liquid chromatography-nuclear magnetic resonance spectroscopy (LC-NMR) is considered an established technique with a number of proven applications for identification of drug metabolites [251-254], natural products [254-257], and environmental contaminants [258] in complex samples. Supercritical fluid chromatography (SFC-NMR) and capillary electrophoresis (CE-NMR) coupled systems have been described, but relatively little used.

Modern magnets for NMR spectroscopy are based on superconducting solenoids with field strengths greater than 400 MHz for ^1H -NMR [2,251,259-262]. The magnetic field is generated by a current circulating in a coil of superconducting wire immersed in a liquid helium Dewar flask. To improve magnet homogeneity an assembly of coils (shim coils) is inserted into the magnet bore. These are designed so that adjustable currents can be fed through them to provide corrections to the magnetic field in any combination of axes. The magnet bore also houses the NMR probe, which contains tunable radio frequency coils for excitation of nuclear spins and detection of the resultant signals. A spectrum is measured by application of a short, intense pulse of radio frequency radiation to excite the nuclei followed by detection of the

induced magnetization in the detector coil as the nuclei relax. The free induction decay (relaxation of the nuclei) is converted to the frequency domain by a Fourier transform. Acquisition of a single ^1H free induction decay (scan) requires a few seconds. To improve the signal-to-noise ratio of the spectrum, excitation pulses are initiated continuously with selected time delays, and a number of sequential scans summed. Some typical experiments require the use of several different pulse types separated by time delays controlled by software. For example, to achieve solvent suppression, to measure low sensitivity nuclei (e.g. ^{13}C , ^{15}N , etc.) by their spin coupling connectivity to protons, to measure nuclear Overhauser enhancement (NOE) effects (for distinction between isomeric structures), to decouple selected internuclear interactions (to assign connectivity), for two-dimensional NMR correlation studies, etc. Modern NMR spectrometers employ integrated computer systems for instrument control, data acquisition, and data analysis. Typical data analysis operations include baseline correction, signal averaging, Fourier transform operations, phase correction, calculation of output peak lists, and plotting spectra.

9.4.1 Chromatographic Interfaces

The development of suitable flow probes and techniques to optimize NMR acquisition conditions laid the foundation for the realization of coupled chromatographic and NMR instruments. Sensitivity enhancements through higher-field magnets, smaller diameter receiver coils, and increased range of receivers, combined with the availability of versatile solvent suppression techniques, have also alleviated many of the initial problems in the design of coupled systems. Nonetheless, the relatively poor mass sensitivity of conventional NMR detection methods, compounded by limited observation time for each chromatographic peak, has remained the primary challenge to continuous flow NMR detection. Flow probe designs represent a compromise between maintaining chromatographic resolution and maximizing NMR mass detection capability. For LC-NMR the volume of the flow cell and connecting tubing is on the order of 0.1 to 0.8 ml, and constitutes a significant source of chromatographic band broadening. These relatively large probe volumes tend to dictate the use of high column flow rates and/or makeup flow that further reduces the peak residence time in the flow cell, and reduces mass sensitivity. In practice, the most straightforward approach to increase mass sensitivity is to extend the observation time for the chromatographic peak in the flow cell by stopped flow operation. Stopped flow operation is also required for two-dimensional correlation experiments based on mutual spin-spin coupling, such as COSY (correlation spectroscopy), TOCSY (total correlation spectroscopy) and NOSEY (nuclear Overhauser effect spectroscopy). Continuous flow operation is generally feasible only when using ^1H or ^{19}F NMR detection, unless isotopically enriched compounds are used.

9.4.1.1 Liquid Chromatography-Nuclear Magnetic Resonance Spectroscopy

The coupling of liquid chromatography to NMR spectroscopy (LC-NMR) requires the use of long column-to-detector transfer lines to place the chromatograph outside the

stray field of the magnet, about 30-50 cm for shielded magnets, and a flow cell (probe) inserted vertically into the bore of the cryomagnet [251-259,263-266]. The flow cell is typically a U-shaped glass tube with an internal diameter of 2-4 mm (detection volumes of 40-250 μ l) inserted in the glass Dewar of a conventional probe body with temperature control. The radio frequency transmitting and receiving coils are fixed to the outside of the glass cell to obtain an optimum filling factor, that is, ratio of the sample volume to the volume inside the detector coil, for maximum sensitivity. Homogeneity of the magnetic field is generally good over the small volume of the flow cells negating the need for spinning. Inverse continuous flow probes contain an additional coaxial coil (matched to the ^{13}C resonance frequency) surrounding the ^1H detection coil for heteronuclear $^1\text{H}/^{13}\text{C}$ shift-correlated experiments.

The detection of weak ^1H -NMR sample signals in the presence of strong solvent signals is impossible due to the limited dynamic range of the receiver coils and A/D converter. In principle, this problem could be circumvented by using fully deuterated solvents for chromatography and spectrum measurements, but this option is generally too expensive for routine applications, and even high purity deuterated organic solvents contain significant amounts of proton-containing impurities that may interfere with the measurement of sample spectra. The exception is deuterium oxide, which is commonly substituted for water in reversed-phase separations. Using deuterium oxide has the added benefit of providing a frequency lock for spectrum measurements and simplifies methods used for solvent suppression. Also, superheated deuterium oxide is a suitable mobile phase for some LC-NMR applications [267].

The frequent use of solvent mixtures and composition gradients in chromatography add to the difficulty of solvent suppression. Low intensity proton resonances associated with ^{13}C -satellites also require suppression. The development of fast, reliable, and powerful solvent suppression pulse techniques, such as WET, have overcome these problems and largely replaced less effective presaturation techniques. WET employs a series of variable tip-angle solvent-selective radio frequency pulses, in which each selective pulse is followed by a dephasing field gradient pulse. WET causes the ^{13}C -satellite peaks to collapse under the central peak, allowing the use of single frequency suppression. The time required for solvent suppression is < 100 ms and high quality spectra free of significant interference are easily obtained by initiating the pulse sequence before each set of scans. When solvent suppression is used, sample signals that reside under the solvent peak are suppressed along with the solvent signal.

The limited spectrum acquisition time restricts continuous flow operation to concentrated samples for most practical applications. Stopped flow is the primary operating mode for most routine applications. An automated valve in the transfer line is used to interrupt the column flow and retain the peak of interest in the flow cell. This allows spectrum measurements to be made over a longer time to enhance the signal-to-noise ratio, as well as enabling a wide range of two-dimensional NMR experiments to be performed. Other common operating modes include peak collection in a series of storage loops (peak picking or peak parking) for subsequent automated off-line spectrum measurements, and time slices, where the column flow is arrested at intervals as the peak

passes through the flow cell. The method of time slices is mainly used for peak purity determinations, or to obtain spectra from incompletely separated compounds. The location of individual peaks for spectrum measurements in the stopped flow mode requires an auxiliary technique to time the arrival of the desired peak at the flow cell. The standard method of peak detection in LC-NMR is in-line UV detection. The apex of the UV peak is used to calculate the time delay for peak transfer, either directly into the flow cell for immediate analysis, or into storage loops for subsequent analysis. Mass spectrometry provides a more specific detection mechanism for compounds lacking a chromophore or masked by co-eluting compounds in the chromatogram (section 9.4.2) [268]. Evaporative light scattering detection is less specific, but also suitable for detecting compounds lacking a chromophore [269]. Software allows automated peak detection for timed transfer of peaks to the flow cell or storage loops.

The detection limits for ^1H -NMR spectra depend on a number of factors. In general, more favorable detection limits are observed for larger flow cell volumes, higher magnetic field strengths, improved fitting factor and quality of detection coils, lower temperatures, shorter receiver bandwidths, and improved preamplifier quality (reduction of noise). For 500 or 600 MHz instruments, ^1H detection limits of about 10–20 μg (observable spectral feature above noise) and minimum identifiable quantity of about 40–100 μg (recognizable spectra above noise) are typical for the continuous flow mode. For the stopped flow mode, detection limits of 5 ng and a minimum identifiable quantity of 50–100 ng with signal averaging overnight, or about 1 μg with signal averaging over 5 min, can be expected. Since detection characteristics depend on a large number of instrument performance parameters that differ between instruments and may be improved upon in the future, these limits should be viewed as qualitative estimates and subject to change.

9.4.1.2 Other Chromatographic Interfaces

Supercritical fluid chromatography is coupled to nuclear magnetic resonance spectroscopy (SFC-NMR) in a manner similar to liquid chromatography [270]. Control of the flow rate and density of the mobile phase is achieved by a backpressure regulator placed after the flow cell. As a consequence the flow cell must be able to withstand the high operating pressures and temperatures ($P > 73$ bar and $T > 305$ K) typically used for separations. A sapphire U-tube heated by circulating hot air around the tube is used for this purpose. Carbon dioxide does not contain any proton signals so the whole ^1H -NMR signal range, can be used for structure identification when organic solvents are not required as a component of the mobile phase for the separation. The main drawback of SFC-NMR is the pressure dependence of ^1H -NMR signals, and the increased spin-lattice relaxation time. Because of this behavior, only modest pressure changes are possible during the acquisition of ^1H -NMR spectra, otherwise severe line broadening will occur.

Several research groups have investigated the use of solenoid and saddle type microcoil transceivers for ^1H -NMR detection in capillary-electromigration separation techniques and open tubular column liquid chromatography [259,265,271]. Either the

separation capillary is inserted through the microcoils (microprobe design) or the microcoils are wrapped directly around a short length of the separation column. The microprobe design is more flexible and facilitates exchange of separation columns, but generally provides lower sensitivity. Either the whole separation column and inlet and outlet buffer vials are housed in the magnet bore or the inlet vial remains outside of the magnet while the rest of the separation system is housed in the magnet bore. The latter approach is more versatile, and allows injection without disturbing the probe position. Shimming is important to counteract signal degradation and line broadening from flow-induced and current-induced changes in the magnetic field homogeneity. Stopped flow operation allows NMR measurements under quiescent conditions where distortions of line width and shape are minimized. Current detection limits are at the low mM level (about 500 ng) at best and further improvements in sensitivity are required for the method to be useful for general applications, where detection limits of about 1 ng are desirable.

9.4.2 Coupled Interfaces with Mass and Infrared Spectrometry

There are clear advantages to acquiring complementary spectral information for structure elucidation as well as savings in time and effort if this information is obtained (nearly) simultaneously and made available for integrated (computer-aided) structure determination. This remains more of a goal than a general reality for compounds separated by liquid chromatography. Systems for LC-UV-NMR-MS are available at present and an extension to LC-UV-NMR-MS-FTIR was achieved with few additional problems [251,254,258,264,267]. These multiple hyphenated systems present a flow and time management problem, and require a mobile phase compatible with the different spectrometers. Non-volatile buffers have to be avoided along with certain additives, such as trifluoroacetic acid, which although ideal for ^1H -NMR spectroscopy may suppress ionization in MS. Formic acid-ammonium formate buffers provide a reasonable compromise for pH control of separations and compatibility with different spectroscopic techniques. Replacing water by deuterium oxide in reversed-phase chromatography simplifies solvent suppression in NMR spectroscopy and is compatible with other spectroscopic techniques. Deuterium oxide will exchange labile protons in common functional groups (e.g. OH, NH_2 , CO_2H , etc.) increasing the mass of the compound and modifying infrared group frequencies. Mixing the eluent with water or methanol prior to MS measurement will re-exchange deuterium for hydrogen. Recording the mass spectrum with and without deuterium exchange allows the number of exchangeable protons to be found. Since continuous flow UV and NMR are non-destructive techniques, their serial coupling to MS (or other spectroscopic techniques) is possible and simplifies system operation. Because of the low sensitivity and the relatively large volume of continuous flow NMR interfaces, however, serial coupling is not common. Parallel coupling to each spectrometer with a series of flow splitters and automated valves positioned after the UV spectrometer provides the most flexible approach. A few percent of the total column flow is directed to

the mass spectrometer with the remainder directed to the NMR, or divided between the NMR and FTIR spectrometers. The significant difference in sensitivity between mass spectrometry and the other spectroscopic techniques requires operation of the latter in the stopped flow mode (NMR) or using a storage interface for off-line measurements (FTIR). Exact timing of peak movements throughout the splitter network is important to locate individual chromatographic peaks in the different spectral databases. The stray field of the NMR magnet dictates the position of other instruments with respect to the magnet. Such multiple hyphenated systems are clearly expensive and complex to operate, but provide a rich source of information, resulting in a more reliable identification of unknown compounds than any of the spectroscopic techniques used alone. Increased automation and integration of software for control and data analysis will make these systems easier to use, even if for the time being they are likely to remain restricted to research laboratories where structure elucidation of unknown compounds in complex mixtures is a primary function.

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Chapter 10

Separation of Stereoisomers

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10.1 INTRODUCTION

Isomers are compounds that have the same molecular formula (constitution) but different structures (configuration). Structural (or configurational) isomers such as *n*-butanol and diethyl ether, for example, often differ appreciably in their physical and chemical properties and, in general, are expected to be relatively easy to separate using conventional chromatographic techniques. Stereoisomers, on the other hand, differ only in the spatial configuration of substituent groups within a molecule (same atomic bond order) and usually have similar physical and chemical properties. Their separation in conventional chromatographic systems can be difficult or impossible without resorting to specific conditions

Several different classes of stereoisomers can be distinguished [1-4]. Conformational isomers (conformers) can be interconverted by rotation about single bonds and correspond to different internal energy minima, for example, the chair and boat conformations of cycloalkanes. These energy minima are generally too small for distinction in chromatographic systems and single peaks are observed. Configurational isomers have significant energy barriers to interconversion and exist as distinct forms that are stable to typical separation conditions. Configurational isomers include geometric isomers, enantiomers, and diastereomers. Geometric isomers owe their existence to hindered rotation about double bonds. The isomers differ in the position of atoms (or groups) relative to a reference plane: in the *cis*-isomer (or *Z* form) the atoms are on the same side; in the *trans*-isomer (or *E* form) they are on opposite sides. Geometric isomers have different physical and chemical properties and can usually be separated, if at times with difficulty, in conventional chromatographic systems.

Enantiomers are stereoisomers that are non-superposable mirror images of each other. Enantiomers are chiral molecules (see Table 10.1 for a description of common terms used to describe different properties of stereoisomers [5-7]). Common examples of enantiomers are molecules containing tetrahedral carbon, silicon, sulfur, or phosphorus atoms bearing four different substituents (or three different substituents and a lone pair of electrons for Group V and VI elements), unsymmetrical sulfoxides, and substituted aziridines. Chirality may also result from the helicity of a macromolecule, such as a protein or polymer. Molecules in which there is a permanent and rigid twist in the planes of atom connectivities display chirality. So do compounds in which a pair of rings is joined at a single common (spiro) atom, when the rings are substituted so as to distinguish between their two faces. Molecules in which two ring systems are joined by a single bond are chiral, if steric effects hinder complete rotation about this bond and the two ring systems are differently substituted, so planes of symmetry are absent. Some examples of the different types of enantiomers are presented in Figure 10.1. Enantiomers have identical physical and chemical properties except for their ability to rotate the plane of polarized light to equal extents but in opposite directions.

Diastereomers (or diastereoisomers) are stereoisomers that are not mirror images of each other. Diastereomers include molecules containing more than one asymmetric (chiral) center and geometric isomers. They have different physical and chemical

Table 10.1
Common terms to describe properties of stereoisomers

Term	Description
Absolute Configuration	The spatial arrangement of the atoms of a chiral molecular entity (or group) and its stereochemical description. The stereochemical arrangement about a particular chiral center is normally assigned using the (<i>R,S</i>)-nomenclature according to the Cahn-Ingold-Prelog rules [7]. The Fischer convention (<i>D,L</i>) based on glyceraldehyde as a reference is considered obsolete. It continues to be used in naming amino acids and carbohydrates, where trivial names are traditional.
Achiral	An object that is superposable on its mirror image
Anomers	Diastereomers of glycosides, hemiacetals, or related cyclic forms of sugars, differing in configuration only at C-1 of an aldose, C-2 of a 2-ketose, etc.
Atropisomers	Result when complete rotation about a single bond in a molecule is prevented by the bulk of non-identical neighboring substituents, so that a pair of enantiomers is formed.
Chirality	The geometric property of a rigid object of being non-superposable on its mirror image; such an object has no symmetry elements, such as a mirror plane, center of inversion, or a rotating-reflection axis.
Chiral Additive	A chiral selector added as a component of a mobile phase or electrophoretic medium.
Chiral Selector	The chiral component of the separation system capable of interacting selectively with the enantiomers to be separated.
Diastereomers	Stereoisomers not related as mirror images. They are characterized by differences in physical properties, and by differences in chemical behavior towards achiral, as well as chiral reagents.
Enantiomer	One of a pair of molecular entities which are mirror images of each other and non-superposable.
Enantiomer Excess	The proportion of one enantiomer in a given mixture of both enantiomers usually expressed as a percent $ee = 100(\chi_R - \chi_S) / (\chi_R + \chi_S)$ where χ_R and χ_S are the mole or weight fraction of the R- and S-enantiomers, respectively, and ee the enantiomer excess.
Racemate	An equimolar mixture of enantiomers. It does not exhibit optical activity.
Stereoisomers	Isomers with an identical constitution but different spatial arrangement of atoms.

properties and can be separated using conventional chromatographic techniques, if at times with difficulty. The formation of diastereomers is the basis of the separation of enantiomers. The diastereomers can be formed by direct interaction with a chiral phase (formation of transient diastereomer association complexes), or after chemical transformation by reaction with a single enantiomer derivatizing reagent.

Enantiomers have identical chemical properties in relation to their reactions with achiral reagents. Their physical properties are identical (e.g. solubility, partition coefficients, boiling points, etc.) So why the interest in enantiomer composition? This arises from the fact that in a chiral environment enantiomers behave as different compounds. The natural world is constructed of chiral systems that employ structure recognition mechanisms as a regulatory function [1,4,8]. The single enantiomers of racemic drugs exhibit differences in their bioavailability, distribution, metabolism, and excretion. It is often the case that one enantiomer is the more active isomer for a given

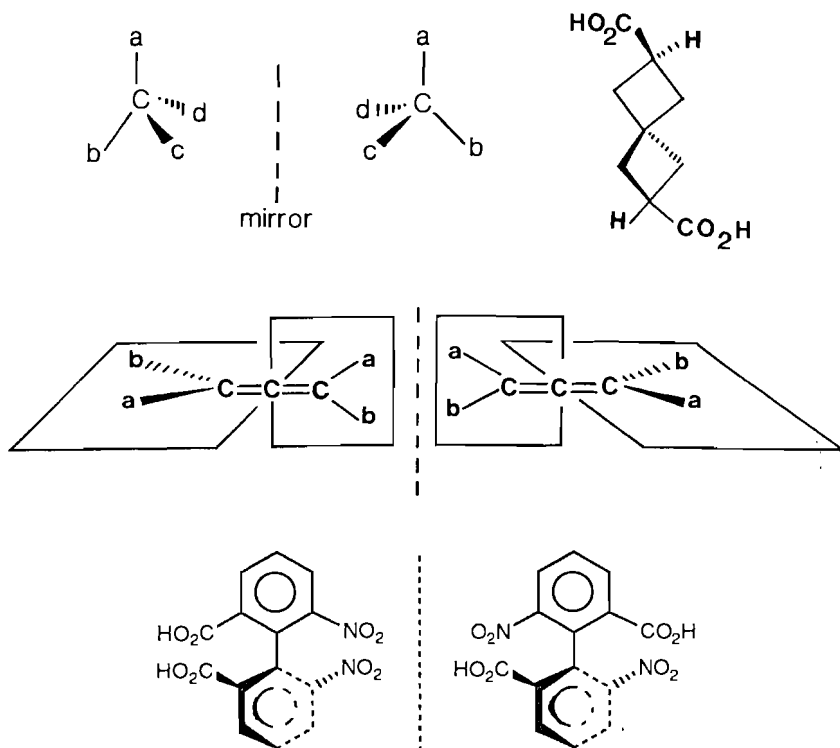


Figure 10.1. Examples of enantiomers. A, an asymmetric carbon atom with four different substituent groups; B, a spiro compound (pair of rings containing different substituents joined at a single [spiro] atom); C, cummulenes (two or more successive double bonds with a rigid twist in the planes of atom connectivities); and D, a molecule in which rotation about a single bond is prevented by the bulk of non-identical neighboring substituents.

action, while the other enantiomer might be even active in a different way, contributing to side-effects, displaying toxicity, or acting as an antagonist. Regulatory authorities now require separate evaluation of single enantiomers throughout the drug discovery process even for products eventually marketed as racemates. Beyond pharmaceutical science, stereochemistry is recognized as a central component of the agrochemical and flavor and fragrance industries. In large part, the interest in the separation of enantiomers has to be seen as unavoidable, to gain an understanding of the properties of biologically active compounds. The success in developing stereoselective separation mechanisms has resulted in new applications exploiting the characteristic properties of enantiomers. For example, in archeology, the measurement of the degree of racemization of specific amino acids is used to date human remains [9]. Enantiomer ratios are used to establish the authenticity and quality of essential oils and flavors [10,11]. Enantiomer labeling, an application of the standard addition technique, is used for the accurate quantification

of enantiomers in complex matrices, in a manner similar to isotopic labeling without the need for mass selective detection [12]. In biological samples, the stability and conversion rates of enantiomers can be quite different, affecting the utility of enantiomer labeling for some sample types and conditions [13].

10.2 ENANTIOSELECTIVITY AND ABSOLUTE CONFIGURATION

The enantioselectivity of a chromatographic system is defined as the preferential interaction with the chiral selector of one enantiomer over the other. It is usually determined as the ratio of the retention factors of two enantiomers in a chiral chromatographic or electrophoretic system. The ratio of the retention factors is equivalent to the chromatographic separation factor (α). It is related to the difference in free energy of the reversible diastereomer association complexes formed by each enantiomer with the chiral selector (section 1.4.4). The separation factor provides a useful practical guide to the suitability of a chromatographic system for the separation of enantiomers. The true enantioselectivity and the chromatographic selectivity, however, are not one and the same thing, and this affects the calculation and interpretation of thermodynamic relationships for enantiomer-chiral selector interactions. The discrepancy arises from the fact that the chromatographic retention factor is composed of contributions from non-specific interactions of the enantiomers with the chromatographic system, which are the same for both enantiomers, as well as specific enantiomer-chiral selector interactions, which are not identical when a separation is obtained [14-16]. The true enantioselectivity will almost always be greater than the ratio of the retention factors and the temperature dependence of the chromatographic selectivity factor, commonly used to evaluate thermodynamic contributions to the retention mechanism, will not be the same as the temperature dependence of the true enantioselectivity. In enantioselective complexation gas chromatography the retention increment can be used to quantitatively differentiate between non-specific and chiral interactions [16], but in other cases isolation of the two contributions to retention is difficult.

Since enantiomers have identical physical and chemical properties, their separation requires a mechanism that recognizes the difference in their shape. A suitable mechanism for chromatography is provided by the formation of reversible transient diastereomer association complexes with a suitable chiral selector. To achieve a useful separation the association complexes must differ in stability resulting from a sterically controlled preference for the fit of one enantiomer over the other with the chiral selector. In addition, the kinetic properties of the formation/dissociation of the complex must be fast on the chromatographic time scale to minimize band broadening and achieve useful resolution. Enantioselectivity based on the formation of transient diastereomer complexes is commonly rationalized assuming a three-point interaction model [1-4,17,18]. Accordingly, enantioselectivity requires a minimum of three simultaneous interactions between the chiral selector and at least one of the enantiomers, where at least one of these interactions is stereochemically dependent. The points of interactions

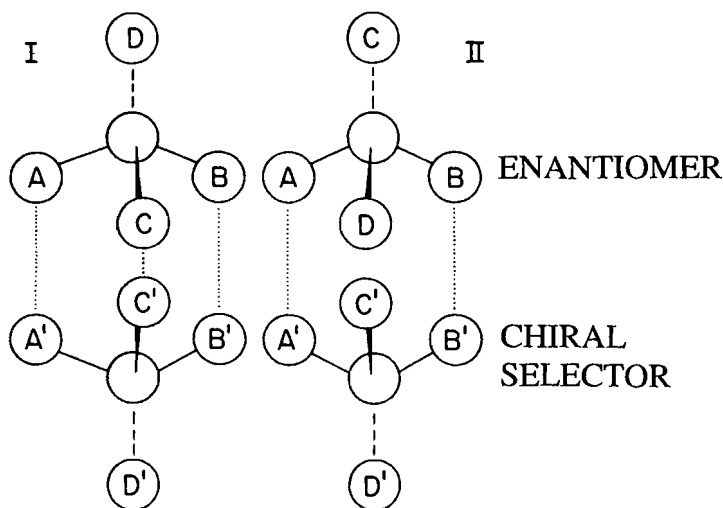


Figure 10.2. Stereoselective formation of diastereomer association complexes between two enantiomers and a chiral selector according to the three-point interaction model.

usually occur by hydrogen bonding, charge transfer (π - π), dipole-type, hydrophobic or electrostatic interactions between substituents of the chiral selector and the enantiomers. For example, in Figure 10.2, enantiomer I forms a three point interaction with the chiral selector represented by the dotted vertical lines while enantiomer II can form only a two point interaction because substituent C is in the wrong configuration to interact with the complementary group of the chiral selector. Enantiomer I, therefore, forms a more stable complex with the chiral selector than enantiomer II, and if the difference in stability of the complexes is sufficient then the two enantiomers will be separated with enantiomer II being less retained. In the modern interpretation of the three-point rule, all the point-to-point interactions do not have to be attractive. Some of them may be repulsive in nature, for example, resulting from steric crowding at the chiral center.

If the chiral selector resides in the stationary phase and all interactions are assumed to be attractive, as in Figure 10.2, then the interactions between groups A and A' and B and B' determine the retention of the weakly bound enantiomer. The third interaction between groups C and C' is responsible for the retention difference and enantioselectivity. In an ideal situation, one would prefer to see the shortest possible retention for the first enantiomer and the largest possible retention for the second enantiomer. This implies that the ideal chiral selector should produce the lowest possible binding energy due to the interactions between groups A and A' and B and B' and the highest possible binding energy for the sterically controlled interaction between groups C and C'. When interactions between groups A and A' and/or B and B' are significantly stronger than between groups C and C' retention is increased without a concomitant change in enantioselectivity. Of course the strength of the individual interactions can

and do vary significantly, and in practice are generally unknown, so the three-point model provides only a convenient framework for explaining enantioselectivity without providing a quantitative tool for its prediction.

The three-point interaction model is successful at explaining enantioselectivity for well-defined chiral selectors, but does not give an accurate description of the chiral recognition mechanism for chiral polymers, also widely used in chromatography to separate enantiomers. An expansion of the original three-point interaction model into a model of molecular chiral recognition can account for these differences [18]. In the model of molecular chiral recognition, binding of the enantiomers to a region of the chiral selector need not involve formation of three simultaneous interactions between the enantiomer and chiral selector. The initial binding of the enantiomers is assumed to result in the formation of diastereomer complexes of equivalent stability. In a subsequent step, the enantiomers and the chiral selector conformationally adjust to each other to maximize the interactions and, thereby, the stability of each of the diastereomer complexes. The conformational adjustment might be driven by several factors other than single point interactions. For example, differences in the steric fit in a topographical feature of the stationary phase, such as a ravine or cavity, and the interdiction of solvent molecules in the binding process providing for the possibility of multipoint interactions in a structured solvent environment. It is the stereochemical dependence of this conformational adjustment step for the initially bound diastereomer complexes that is responsible for the difference in stability of the complexes, and therefore, separation of the enantiomers.

The probability of separating a pair of enantiomers in a particular system is not easy to predict from their structure. Lack of knowledge of the geometry of the solute accessible binding structures in the chiral stationary phases, and the multiple types of cavities involved, has to date hindered a detailed understanding of the chiral discrimination process at the molecular level. The success rate in separating enantiomers is quite high in practice owing to the large number of chiral stationary phases available, and the high efficiency of chromatographic systems, that provide adequate separations with very small separation factors. The selection of the initial separation conditions remains an empirical process, generally made with scant regard for the chiral recognition mechanism. Commercial databases, such as CHIRBASE and CHIRSOURCE, containing about 61,000 separations from some 40,000 bibliographic entries (2001) can provide a useful starting point [19,20]. These databases are searchable by either two- or three-dimensional structures and substructures, indicating separation conditions suitable for related compounds when the input structure is absent from the database. NMR, and to a lesser extent X-ray crystallography, has been used to provide direct evidence for the binding of enantiomers to chiral selectors and for optimizing chiral selectors in the development of new stationary phases [8,21,22]. Quantitative structure enantiomer-retention relationships (QSERRs) have been developed to predict enantioselectivity but with only limited success [8,23]. These models provide some insight into the general importance of different binding interactions at the chiral selector, but have not proven useful for identification of

chiral selectors for particular separations. The main problem is identifying suitable molecular descriptors for the enantiomers that adequately reflect their chemical and structural properties at the detail required for useful predictions. Atomistic chiral recognition models based on molecular mechanics may provide a suitable approach for predicting enantioselectivity in the future, but so far have provided qualitative information only, suitable for establishing the elution order of a limited set of closely related compounds [24,25].

Chromatographic methods are suitable for the determination of the absolute configuration of enantiomers when only limited sample amounts are available. The sample need not be pure, if the separation system is able to separate the enantiomers of interest from interfering compounds. The determination of absolute configuration is straightforward when authentic standards for the single enantiomers are available. The enantiomer configuration is identified by co-elution, and should be confirmed by the reversal of the elution order on the same stationary phase with the opposite configuration of the chiral selector, when available. In principle, the absolute configuration of chiral samples can be determined in the absence of single enantiomers after calibration of the separation system with related reference compounds of known stereochemistry. Assignment of absolute configuration by analogy, however, is suspect, if sometimes adopted for pragmatic reasons. As a minimum, comparisons should be made at identical temperatures for gas chromatography and mobile phase compositions for other methods. The elution order of enantiomers is strongly influenced by temperature in gas chromatography and an inversion of the elution order is possible at temperatures either side of the isoelution temperature (determined in a van't Hoff plot as the temperature at which the enthalpy and entropy contribution cancel each other) [16]. In liquid chromatography temperature and mobile phase composition can affect the elution order through changes in the conformation of the stationary phase or competition at the chiral selector-binding site [26,27].

10.3 SEPARATION OF ENANTIOMERS

There are two general approaches for the separation of enantiomers [1-4,28-32]. The direct method is based on the formation of transient diastereomer association complexes with a chiral selector immobilized in the stationary phase, or added to the mobile phase. The former approach requires the use of special stationary phases (section 10.4) while the later uses conventional stationary phases with special additives included in the mobile phase (section 10.5). When preparative applications are contemplated the use of immobilized chiral selectors is the more common approach. Method selection also depends on the choice of the separation mode, Table 10.2. While chiral stationary phases are the only choice for gas chromatography [16,28,33-38], and are used almost exclusively for supercritical fluid chromatography [39-43] and capillary electrochromatography [44-47], they also dominate the practice of liquid chromatography

Table 10.2

Common methods for separating enantiomers by formation of transient diastereomer complexes

Technique	Selector	Type
Gas Chromatography	Stationary phase	Amino Acid Derivatives Metal Chelates
Liquid Chromatography	Stationary phase	Cyclodextrin Derivatives Amino Acid Derivatives Low-Mass Synthetic Selectors Poly(saccharide) Derivatives Cyclodextrin Derivatives Glycopeptides Metal Chelates Proteins
		Helical Polymers
		Cyclodextrin Derivatives
		Metal Chelates
		Amino Acid Derivatives
		Proteins
	Mobile phase	Cyclodextrin Derivatives Metal Chelates Amino Acid Derivatives Proteins
Thin-Layer Chromatography	Stationary phase	Metal Chelates Poly(saccharide) Derivatives
	Mobile phase	Cyclodextrin Derivatives Proteins Amino Acid Derivatives
		Polysaccharide Derivatives
		Cyclodextrin Derivatives
Supercritical Fluid Chromatography	Stationary phase	Glycopeptides Low-Mass Synthetic Selectors Metal Chelates
		Cyclodextrin Derivatives
		Polysaccharide Derivatives
		Amino Acid Derivatives
Capillary Electrophoresis	Mobile phase	Glycopeptides
		Proteins
		Metal Chelates
		Poly(saccharides)
		Chiral Surfactants (MEKC)
		Cyclodextrin Derivatives
Capillary Electrochromatography	Stationary phase	Glycopeptides
		Cyclodextrin Derivatives
		Poly(saccharide) Derivatives

[4,5,28-32,47,48]. Mobile phase chiral additives are dominant in capillary electrophoresis [49-57], and are preferred for thin-layer chromatography [58-60]. These techniques consume relatively modest amounts of mobile phase allowing expensive chiral additives to be used economically. Other general considerations in choosing a separation system are the volatility and solubility of the enantiomers and the efficiency of the chromatographic system. For thermally stable and volatile enantiomers, gas chromatography is the preferred technique, because its superior efficiency results in the baseline separation of enantiomers with very small separation factors. For water-soluble enantiomers of low volatility, liquid chromatography is the most important technique, owing to the availability of a wide range of stationary phases with complementary selectivity.

Liquid chromatography also provides easy scale-up for preparative applications. When only analysis is required, capillary electrophoresis provides an alternative choice, that is gaining in popularity because of its higher efficiency, although liquid chromatographic methods are considered more robust and more reliable for the quantification of minor enantiomers. For enantiomers of low water solubility and low volatility normal-phase liquid chromatography, supercritical fluid chromatography, and non-aqueous capillary electrophoresis are generally used. Traditionally, normal-phase liquid chromatography is the dominant technique for the separation of these enantiomers, but supercritical fluid chromatography is gaining increasing support because it offers higher efficiency and faster separations. Thin-layer chromatography provides an inexpensive and flexible approach for the separation of enantiomers, but is limited by low separation efficiency. Capillary electrochromatography offers higher efficiency than liquid chromatography with the same stationary phases, and has a higher loading capacity than capillary electrophoresis. It has considerable potential for enantiomer separations that may be realized more fully with on-going improvements in column and instrument technology (section 8.4).

The second, and less popular approach for the chromatographic separation of enantiomers, is the indirect method (section 10.7). Separation is possible using conventional chromatographic systems after reaction of the enantiomers with a single enantiomer derivatizing reagent to form a pair of covalently bonded diastereomers. This method requires that the enantiomer contain a suitable functional group for the reaction. Indirect methods generally require more effort for validation than direct methods because of concerns over additional sources of error (enantiomer purity and stability of the derivatizing reagent, differences in reaction rates for the two enantiomers, different detector responses for the diastereomers, etc.) The direct method can be applied to enantiomers lacking reactive functional groups, and is not limited by the need for reagents of high enantiomeric purity. Although the extent of resolution on a chiral stationary phase will depend on the enantiomeric purity of the phase, separations are still possible with phases exhibiting reasonable enantiomer excess. However, there is no universal chiral phase for the separation of all enantiomers, and therefore, both the direct and indirect method of separation has their uses for specific applications.

10.4 CHIRAL STATIONARY PHASES

Over 100 stationary phases with immobilized chiral selectors are commercially available with further stationary phases described in the literature. Any attempt at a general classification into a reasonable number of groups with similar properties is not easy. Probably the most widely adopted classification scheme was proposed by Wainer, and is loosely based on the chiral recognition mechanism, Table 10.3 [61]. In the following sections, a different approach has been adopted based on the type of chiral selector. Coverage is incomplete, but includes all of the widely used stationary phases.

Table 10.3

Classification of immobilized chiral selectors (chiral stationary phases) by enantioselectivity mechanism

Type	Description
I	Diastereomer complexes formed by attractive interactions (e.g. hydrogen bonding, π - π , dipole-type interactions) between enantiomers and chiral selector. Examples: low-mass synthetic selectors, amino acid derivatives, glycopeptides
II	Primary mechanism of diastereomer complex formation is through attractive interactions (Type I) but inclusion complexes also play a role. Example: poly(saccharide) derivatives.
III	Formation of inclusion complexes is important but secondary attractive interactions also play a role. Examples: cyclodextrin derivatives, helical polymers and crown ethers
IV	Formation of diastereomer ternary complexes involving a transition metal ion and a single enantiomer ligand (usually an amino acid) Examples: Cu(II)-L-hydroxyproline, Mn(II) <i>bis</i> [3-heptafluorobutanoyl]-(1R)-camphorate]
V	Formation of diastereomer complexes by a combination of hydrophobic, electrostatic, and hydrogen-bonding interactions with a protein. Examples: bovine and human serum albumin, α_1 -acid glycoprotein, enzymes

10.4.1 Cyclodextrin Derivatives

Cyclodextrins are natural macrocyclic oligosaccharides containing six (α -), seven (β -), or eight (γ -) D-glucose monomers in a chair conformation connected via α -(1,4)-linkages, Figure 10.3 [3,28-30,33,62-67]. The glucose rings are arranged in the shape of a hollow truncated cone with a relatively hydrophobic cavity and a polar outer surface where the hydroxyl groups are located. The larger opening of the cone is surrounded by the secondary (C-2 and C-3) hydroxyl groups, while the primary (C-6) hydroxyl groups are located at the smaller end of the cone. The primary (C-6) hydroxyl groups are free to rotate and can partially block the smaller entrance to the cavity. The restricted conformational freedom and orientation of the secondary hydroxyl groups encircling the opposite end of the cavity are thought to play an important role in the enantioselectivity of cyclodextrins. Derivatizing the outer rim hydroxyls with various functional groups offers a simple mechanism to modify the properties of the natural cyclodextrins, expanding their application range for enantiomer separations, and to optimize their physical properties for use as stationary phases. A wide range of derivatized cyclodextrins is currently used as stationary phases for gas, liquid, and supercritical fluid chromatography, and as mobile phase additives in capillary electrophoresis and thin-layer, and liquid chromatography (section 10.5).

10.4.1.1 Gas Chromatography

Initial attempts to use cyclodextrins and their derivatives as chiral stationary phases in gas chromatography met with limited success owing to their unfavorable melting points or decomposition temperatures [28,30,33-36]. This changed with the discovery that some peralkylated derivatives (e.g. pentyl), partially alkylated derivatives, and mixed alkylated and acylated derivatives were viscous liquids that could be coated on deactivated glass surfaces, Table 10.4 [68-73]. In the absence of oxygen contamination of the

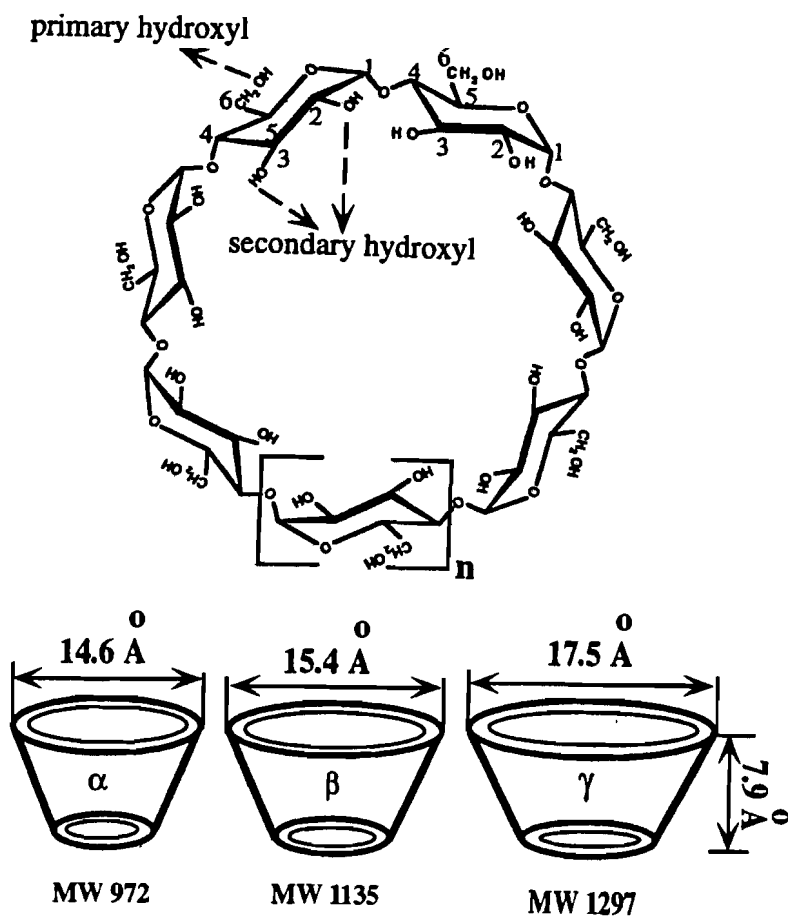


Figure 10.3. Structures of natural cyclodextrins indicating position of hydroxyl groups and cone dimensions. α -Cyclodextrin ($n = 0$), β -cyclodextrin ($n = 1$) and γ -cyclodextrin ($n = 3$).

carrier gas these phases have good thermal stability, and can be used at temperatures up to about 200°C. Some 85% of enantiomers separated by gas chromatography on derivatized cyclodextrins can be separated on one of three derivatized cyclodextrins: octakis(3-*O*-trifluoroacetyl-2,6-di-*O*-*n*-pentyl)- γ -cyclodextrin; heptakis(di-*O*-methyl)- β -cyclodextrin; and heptakis(2,6-di-*O*-*n*-pentyl)- β -cyclodextrin. Octakis(3-*O*-butyryl-2,6-di-*O*-*n*-pentyl)- γ -cyclodextrin is also stated to have a broad application range. It is generally accepted that enantioselectivity results from either inclusion formation and/or surface interactions occurring to different extents for the various cyclodextrin derivatives [73,74]. Although one phase may provide a better separation than others may for a particular enantiomer, several phases will often providing some separation for the

Table 10.4

Some cyclodextrin derivatives used as chiral selectors in gas chromatography
(Stationary phases commercially available on open tubular columns)

Cyclodextrin derivative	MAOT ¹
<i>(i) Viscous liquids</i>	
Hexakis(2,3,6-tri- <i>O</i> -n-pentyl)- α -cyclodextrin	180
Hexakis(3- <i>O</i> -acetyl-2,6-di- <i>O</i> -n-pentyl)- α -cyclodextrin	200
Hexakis(3- <i>O</i> -trifluoroacetyl-2,6-di- <i>O</i> -n-pentyl)- α -cyclodextrin	180
Hexakis(2,6-di- <i>O</i> -n-pentyl)- α -cyclodextrin	200
Hexakis(<i>O</i> -(<i>S</i> -2-hydroxypropyl)-per- <i>O</i> -methyl)- α -cyclodextrin (mixture)	200
Heptakis(2,3,6-tri- <i>O</i> -n-pentyl)- β -cyclodextrin	200
Heptakis(2,3,6-tri- <i>O</i> -methyl)- β -cyclodextrin	230
Heptakis(3- <i>O</i> -acetyl-2,6-di- <i>O</i> -n-pentyl)- β -cyclodextrin	200
Heptakis(3- <i>O</i> -trifluoroacetyl-2,6-di- <i>O</i> -n-pentyl)- β -cyclodextrin	200
Heptakis(di- <i>O</i> -methyl)- β -cyclodextrin (mixture)	230
Heptakis(2,6-di- <i>O</i> -n-pentyl)- β -cyclodextrin	200
Heptakis(<i>O</i> -(<i>S</i> -2-hydroxypropyl)-per- <i>O</i> -methyl)- β -cyclodextrin (mixture)	200
Octakis(3- <i>O</i> -trifluoroacetyl-2,6-di- <i>O</i> -n-pentyl)- γ -cyclodextrin	180
Octakis(3- <i>O</i> -propyl-2,6-di- <i>O</i> -n-pentyl)- γ -cyclodextrin	200
Octakis(3- <i>O</i> -butyl-2,6-di- <i>O</i> -n-pentyl)- γ -cyclodextrin	200
Octakis(2,6-di- <i>O</i> -n-pentyl)- γ -cyclodextrin	200
Octakis(<i>O</i> -(<i>S</i> -2-hydroxypropyl)-per- <i>O</i> -methyl)- γ -cyclodextrin (mixture)	200
<i>(ii) Cyclodextrin derivatives dissolved in a poly(siloxane)²</i>	
Hexakis(2,3,6-tri- <i>O</i> -methyl)- α -cyclodextrin	250
Hexakis(2,3- <i>O</i> -dimethyl-6- <i>O</i> - <i>t</i> -butyldimethylsilyl)- α -cyclodextrin	260
Hexakis(2,3- <i>O</i> -diacetyl-6- <i>O</i> - <i>t</i> -butyldimethylsilyl)- α -cyclodextrin	230
β -Cyclodextrin	230
Heptakis(2,3,6-tri- <i>O</i> -methyl)- β -cyclodextrin	250
Heptakis(3- <i>O</i> -n-pentyl- <i>O</i> -2,6-dimethyl)- β -cyclodextrin	250
Heptakis(2,3- <i>O</i> -dimethyl-6- <i>O</i> - <i>t</i> -butyldimethylsilyl)- β -cyclodextrin	260
Heptakis(2,3- <i>O</i> -diethyl-6- <i>O</i> - <i>t</i> -butyldimethylsilyl)- β -cyclodextrin	230
Heptakis(2,3- <i>O</i> -dipropyl-6- <i>O</i> - <i>t</i> -butyldimethylsilyl)- β -cyclodextrin	230
Heptakis(2,3- <i>O</i> -diacetyl-6- <i>O</i> - <i>t</i> -butyldimethylsilyl)- β -cyclodextrin	230
Octakis(2,3,6-tri- <i>O</i> -methyl)- γ -cyclodextrin	250
Octakis(2,3- <i>O</i> -dimethyl-6- <i>O</i> - <i>t</i> -butyldimethylsilyl)- γ -cyclodextrin	260
Octakis(2,3- <i>O</i> -diacetyl-6- <i>O</i> - <i>t</i> -butyldimethylsilyl)- γ -cyclodextrin	230

¹Highest temperature (°C) for isothermal operation.

²Most common poly(siloxane) solvents are poly(cyanopropylphenyldimethylsiloxane) with 14% cyano-propylphenylsiloxane monomer or poly(dimethyldiphenylsiloxane) with 20 to 35 % diphenylsiloxane monomer. Mixing ratio for the cyclodextrin derivative is 5-50% (w/w).

same enantiomers. So far no simple method has emerged to identify the optimum stationary phase for a separation from enantiomer structure. As well as separation of the natural enantiomers, achiral derivatives can be used to enhance resolution (e.g. separation of enantiomeric amines and alcohols as trifluoroacetyl derivatives) and to allow separation at lower temperatures, which are generally more favorable for enhancing enantioselectivity.

The general use of cyclodextrin derivatives as stationary phases has declined in recent years in favor of cyclodextrin derivatives either dissolved in, or chemically bonded to, a poly(siloxane) stationary phase. These phases generally possess higher thermal and mechanical stability, as well as higher efficiency. A wider range of cyclodextrin derivatives can be employed, since melting point and phase transition are no longer considerations for solutions, Table 10.4 [30,33-38]. The cyclodextrin derivatives are typically dissolved in moderately polar viscous poly(siloxane) stationary phases at a concentration of 5-50 % (w/w). The coating characteristics of the column resemble those of the bulk solvent, which can be varied to reduce retention and elution temperatures [75]. Both retention and enantioselectivity is influenced by the choice of poly(siloxane) solvent and the concentration of cyclodextrin derivative. It is generally observed that the enantioselectivity does not increase linearly with the concentration of the cyclodextrin derivative, and optimum values are often found in the low to intermediate concentration range (< 30% w/w for β -cyclodextrin derivatives).

In general terms, the ring size and substituents bound through the C-2, C-3 and C-6 hydroxyl groups significantly influence the physical properties and enantioselectivity of the cyclodextrin derivatives [76-81]. Alkyl and acetyl substituents at C-2 and C-3 contribute significantly to the enantioselectivity of the cyclodextrin derivatives. There is some evidence that the substituent at C-2 is more important when the chiral recognition mechanism involves interactions at the outer surface of the cyclodextrin and the substituent at C-3 when inclusion dominates the chiral recognition mechanism. These considerations are based largely on the relative orientation of the C-2 and C-3 substituents at the larger rim of the cyclodextrins. Substituents at the C-6 position play a significant role in determining the physical and chemical properties of the cyclodextrin derivative (melting point, solubility, etc.) They may also influence enantioselectivity by blocking the entrance at the smaller rim, and by modifying the conformation of the cyclodextrin. The α - and β -cyclodextrins with 6-*O*-*t*-butyldimethylsilyl ether (or 6-*O*-*t*-hexyldimethylsilyl ether) groups with methyl or acetyl groups at C-2 and C-3 are among the most effective cyclodextrin derivatives for the separation of different enantiomer types [82-90].

Improvements in column technology have been achieved by incorporating the cyclodextrin into a poly(dimethylsiloxane) polymer that is suitable for thermal immobilization (section 2.3.4). Cyclodextrin derivatives with a monooctamethylene spacer arm attached to either the C-6 or C-2 hydroxyl group of a single glucose monomer can be incorporated into a poly(dimethylmethylhydrosiloxane) polymer by the hydrosilylation reaction, Figure 10.4 [91-96]. Some columns of this type are commercially available [e.g. heptakis(2,3,6-tri-*O*-methyl)- β -cyclodextrin (Chirasil-Dex), heptakis(3-*O*-trifluoroacetyl-2,6-di-*O*-*n*-pentyl)- β -cyclodextrin and octakis(3-*O*-butyryl-2,6-di-*O*-*n*-pentyl)- γ -cyclodextrin (Chirasil- γ -Dex)], although columns containing dissolved selectors remain the most widely used. The advantages of immobilization are: an improvement in efficiency, inertness, and stability; compatibility with all injection techniques; the possibility of preparing columns with a higher concentration of the chiral selector; and the use of a non-polar poly(dimethylsiloxane) matrix (in which cyclodextrin deriva-

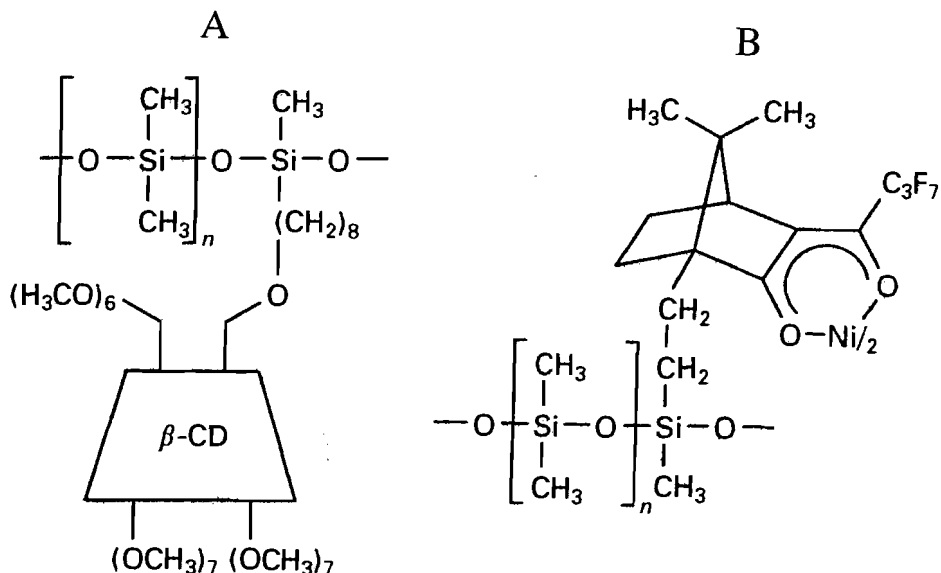


Figure 10.4. Structures of immobilized poly(siloxane) chiral stationary phases containing (A) a cyclodextrin derivative (Chirasil-Dex) and (B) a metal complex (Chirasil-Nickel).

tives have poor solubility) resulting in lower elution temperatures for polar enantiomers. These columns are also suitable for applications using open tubular column supercritical fluid chromatography, liquid chromatography, and capillary electrochromatography [38,93-96].

The rationalization of chiral recognition involving cyclodextrin derivatives is difficult, since almost all classes of enantiomers are susceptible to separation on a certain cyclodextrin derivative, often with no logical dependence on molecular shape, size, or functionality. The high efficiency of open tubular columns allows small selectivity factors to be exploited to yield a useful separation. This contributes just as much to the success of the cyclodextrin phases in gas chromatography as does their enantioselectivity, which can be poor, and yet an acceptable separation obtained. The ability of cyclodextrins to discriminate between enantiomers of unfunctionalized analytes, such as hydrocarbons, implies that the formation of inclusion complexes with the chiral cavity of the cyclodextrin derivatives is important. Variations in the separation properties with cavity size tend to support this general assumption. It is also clear that enantiomer separations occur under conditions where inclusion complexation is unlikely implying that formation of an inclusion complex is not a prerequisite for enantiomer discrimination. Cyclodextrin derivatives have been spectacularly successful in separating a wide range of low molecular mass alcohols, amines, amino acids, epoxides, carboxylic acids, esters, lactones, ethers, haloalkanes, and hydrocarbon enantiomers. Many of these compounds are difficult or impossible to separate by other means, and are economically important

in evaluating the properties of essential oils, food additives, flavors, and chiral synthetic building blocks for more complex molecules [30,33,37,38].

10.4.1.2 Liquid Chromatography

Cyclodextrins and their derivatives can be covalently bonded to silica gel via a spacer arm using any of three general approaches [30,63,97-99]. The silica surface is first reacted with a reagent containing a spacer arm with a terminal functional group that can react with a free hydroxyl group of the cyclodextrin; the cyclodextrin is substituted with a spacer arm containing a terminal functional group that reacts with the silanol groups of the silica surface; or both the cyclodextrin and the silica support are functionalized with substituents that react to form the spacer arm. A wide range of chemistries is employed for these reactions, and in general, the approach used for the preparation of commercially available columns has not been disclosed. Due to the spatial requirements of the bulky cyclodextrins, surface coverage is typically relatively low ($\approx 80\text{--}120\ \mu\text{mol/g}$). In general, unreacted silanol groups and surplus surface bound groups used for the immobilization of the cyclodextrins, influence the enantioselectivity of the column packing.

Immobilized cyclodextrins and their derivatives are used in three general modes for liquid chromatography [28-30,32,63,66,99-102]. Cyclodextrins are used primarily in the reversed-phase or polar organic solvent mode. Acylated α -, β - and γ -cyclodextrins and 2-hydroxypropyl, 1-naphthylethyl carbamate, 3,5-dimethylphenyl carbamate and 4-toluyyl derivatives of β -cyclodextrin were introduced for normal-phase separations originally, but are now used for reversed-phase separations as well. Inclusion complex formation is believed to be significant only when the native or derivatized cyclodextrins are used in the reversed-phase mode. Only if the less polar (preferably aromatic) part of the analyte fits tightly into the cyclodextrin cavity and functional groups close to the stereogenic center interact with the chiral exterior (hydroxyl groups of the glucose units), can enantiomer separations be expected. The tighter the fit of the guest molecule the better the separation. Small molecules that fit loosely into the cavity are not resolved. In general, analytes with substituted phenyl, naphthyl, and biphenyl rings can be separated on β -cyclodextrin and analytes with 3 to 5 rings in their structure, on γ -cyclodextrin. Retention and enantioselectivity depends on the pH, ionic strength, type of organic modifier and its concentration, and temperature. The pH and ionic strength of the mobile phase has most effect on enantiomers containing ionizable functional groups, is difficult to predict, and small changes can have a significant influence on enantioselectivity. Typical buffer concentrations are 10-200 mM of triethylamine phosphate, ammonium nitrate, ammonium acetate, or sodium citrate. Methanol and acetonitrile are the most commonly used organic modifiers, but ethanol and propan-1-ol are more efficient at displacing strongly retained analytes.

Reaction of the cyclodextrin with single enantiomer reagents introduces new chiral selector sites into the cyclodextrin suitable for enantiomer separations in the normal-phase mode. Inclusion complex formation is assumed to play a minor role (the least polar component of the mobile phase occupies the cavity thereby preventing inclusion

complex formation). Instead, enantioselectivity results from the stereoselective binding to the external functional groups of the (partially) derivatized cyclodextrin by hydrogen bonding, dipole-type and π - π interactions. Since inclusion complex formation is not important, cavity size is not a significant factor for stationary phase selection, while the choice of derivative is. Separations are usually performed with hexane containing various amounts of ethanol or propan-2-ol as the mobile phase. The normal-phase mode is suitable for the separation of enantiomers of low water solubility, or water-soluble enantiomers after derivatization. The separation mechanism is similar to the poly(saccharide) derivatives (section 10.4.2), which often provide better resolution in the normal-phase mode.

In the polar organic solvent mode, the mobile phase is usually acetonitrile with small amounts of hydrogen-bonding modifiers, such as methanol, acetic acid, and triethylamine. Inclusion complex formation is suppressed by acetonitrile while at the same time promoting hydrogen bonding between hydroxyl groups on the cyclodextrin rim with suitable enantiomers. At least two functional groups must be present in the analyte for effective separation, and at least one of these functional groups must be on or near the stereogenic center for the analyte. The chiral recognition mechanism is thought to involve interactions at the rim of the cyclodextrin cavity, organizing the analytes as a cover over the entrance to the cavity. Hydrogen bonding and dipole-type interactions, together with steric factors, are all thought to be important for this interaction. Most of the enantiomers separated in the polar organic solvent mode contain amine groups, while a few have carboxylic acid or phenol groups. Separations are usually optimized by varying the amount of acetic acid and triethylamine, or the ratio of acetic acid to triethylamine, such that the combined acid/base concentration is in the range 0.002 to 2.5% (v/v), in a mobile phase containing 85-100% acetonitrile and 15-0% methanol. Addition of methanol reduces the retention time of strongly retained analytes.

Crown ethers, such as 18-crown-6 and its derivatives and calixarenes also form host-guest complexes. Crown ether derivatives are suitable for the separation of enantiomers containing a primary or secondary amine group near a stereogenic center using reversed-phase liquid [103-105] or gas [106] chromatography. Currently, a crown ether stationary phase containing binaphthyl atropisomers as chiral units with an 18-crown-6 backbone is commercially available for liquid chromatography (Crownpak CR). This phase binds ammonium ions stereoselectively by inclusion complex formation and multiple hydrogen bonding interactions between the ammonium ion and the oxygens of the crown ether. Enantioselectivity results from steric interactions at the binding site. The application range is limited by the specific nature of the chiral recognition mechanism.

10.4.2 Poly(saccharide) Derivatives

The ester and carbamate derivatives of cellulose and amylose are among the most successful and versatile chiral stationary phases for liquid and supercritical fluid chromatography [1,4,28,107-109]. These phases are prepared by reaction of the poly(saccharide) with an acid chloride (ester derivative) or phenylisocyanate (carbamate

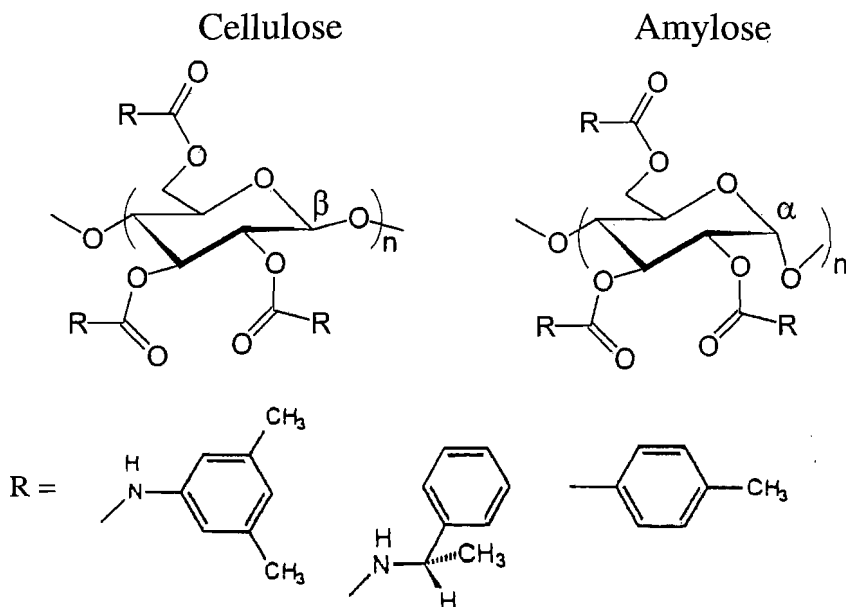


Figure 10.5. General structures of the cellulose and amylose derivatives. Additional R groups are identified in Table 10.5.

derivative). The derivatized poly(saccharides) are then coated from solution onto a wide pore 3-aminopropylsiloxane-bonded silica support by solvent evaporation. The general structure of the cellulose and amylose derivatives is shown in Figure 10.5. Commercially available columns are summarized in Table 10.5. Because these stationary phases are physically coated, the solubility or swelling of the stationary phase restricts the range of solvents that can be used as a mobile phase. To improve the stability of the stationary phases, a number of methods have been described to immobilize the poly(saccharide) derivatives on silica-based supports, including coupling reactions with bifunctional reagents, radical crosslinking, or reaction of the terminal group of the poly(saccharide) with the amino terminal group of the support [108-110]. Immobilization was found to significantly alter the enantioselectivity of the stationary phase, often, but not always, leading to lower resolution. These phases have been little used in practice. Microcrystalline cellulose triacetate, which has a different physical form and enantioselectivity to the precipitated cellulose ester stationary phases, is widely used in medium-pressure liquid chromatography for preparative-scale separations, as a relatively low cost and versatile chiral stationary phase. The helical structure of the microcrystalline cellulose phases contributes significantly to their enantioselectivity by providing a cavity for inclusion, which is absent in the precipitated cellulose phases. In the latter case, hydrogen bonding, π - π , and dipole-type interactions at the polymer surface dominate the chiral recognition mechanism.

Table 10.5

Chiral stationary phases prepared from silica-based coated poly(saccharide) derivatives

Poly(saccharide)	Derivative	Designation*
<i>(i) Esters</i>		
Cellulose	triacetate	Chiralcel OA
	tribenzoate	Chiralcel OB
	tris(4-methylbenzoate)	Chiralcel OJ
	tricinamate	Chiralcel OK
<i>(ii) Carbamates</i>		
Cellulose	tris(phenyl carbamate)	Chiralcel OC
	tris(3,5-dimethylphenyl carbamate)	Chiralcel OD
	tris(4-methylphenyl carbamate)	Chiralcel OG
	tris(4-chlorophenyl carbamate)	Chiralcel OF
	tris(3,5-dimethylphenyl carbamate)	Chiralpak AD
Amylose	tris(1-phenylethyl carbamate)	Chiralpak AS

* Trademark of Daicel Chemical Industries. -H indicates the poly(saccharide) derivative is coated on a 5 μm particle support (usual particle size 10 μm) and -R that the poly(saccharide) derivative is intended for use in reversed-phase liquid chromatography.

A wide range of alcohols, carbonyl compounds (particularly if the carbonyl group is close to the stereogenic center), lactones, phosphorus compounds, aromatic compounds, and sulfoxides have been successfully resolved on the different poly(saccharide) derivatives. Non-polar mobile phases are commonly used, although not exclusively, and the selectivity of the various cellulose polymers changes with the character of the ester or carbamate groups used to derivatize the polymer.

10.4.2.1 Liquid Chromatography

The popularity of the poly(saccharide) derivatives as chiral stationary phases is explained by the high success rate in resolving low molecular mass enantiomers. It has been estimated that more than 85% of all diversely structured enantiomers can be separated on poly(saccharide) chiral stationary phases, and of these, about 80% can be separated on just four stationary phases. These are cellulose tris(3,5-dimethylphenyl carbamate), cellulose tris(4-methylbenzoate), amylose tris(3,5-dimethylphenyl carbamate), and amylose tris(1-phenylethyl carbamate). Typically, n-hexane and propan-2-ol or ethanol mixtures are used as the mobile phase [111]. Both the type and concentration of aliphatic alcohols can affect enantioselectivity. Further mobile phase optimization is restricted to solvents compatible with the stationary phase, such as ethers and acetonitrile, as binary or ternary solvent mixtures, but generally not chloroform, dichloromethane, ethyl acetate, or tetrahydrofuran. Small volumes of acidic (e.g. trifluoroacetic acid) or basic (n-butylamine, diethylamine) additives may be added to the mobile phase to minimize band broadening and peak tailing [112]. These additives, however, may be difficult to remove from the column by solvent rinsing to restore it to its original condition.

The derivatized cellulose and amylose stationary phases also shows high enantioselectivity in the reversed-phase mode [113-115]. The poly(saccharide) derivatives have

no ionic sites, and to obtain adequate retention of ionized compounds, it is necessary to maintain all analytes in a neutral form using ion suppression or ion-pair formation. For basic analytes, pH selection is restricted by the solubility of the silica support (pH < 7-9), and ion-pair reagents, such as perchlorate or phosphorous hexafluoride, are commonly used. The enantioselectivity often depends on the concentration and identity of the ion-pair reagent.

The chiral recognition mechanism of poly(saccharide) derivatives appears to depend on the conformation of the polymer chain and the structure of the substituents introduced by derivatization. Cellulose and amylose are glucose polymers with a different connection between the monomer units (1,4- α or 1,4- β link) resulting in a helical structure with different enantioselectivity. A reversal of the elution order of enantiomers on cellulose and amylose derivatives is not unusual. The chiral recognition mechanism for carbamate derivatives is influenced by the substituents on the phenyl group, since the substituents modify the polarity of the carbamate group. The hydrogen bond donor and acceptor sites of the carbamate are arranged close to the core of the helical polymer axis of the glucose units forming helical grooves. These can direct the interaction and selective insertion of enantiomers with complementary binding sites, by directional hydrogen bonding and dipole-type interactions. In addition, the aromatic groups of the enantiomers may undergo π - π interactions with the phenyl groups of the polymer, which are located on the exterior of the groove.

Polymerization of methacrylates and (meth)acrylamides, in the presence of a chiral anion catalyst results in the formation of a polymer with a helical structure, if the ester side chains contain a bulky group [116]. These polymers can be physically coated onto macroporous silica for liquid chromatography. Enantioselectivity in this case results from insertion and fitting of the analyte into the helical cavity, similar to the poly(saccharide) derivatives. Aromatic compounds, and molecules with a rigid non-planar structure, are often well resolved on this phase. The triphenylmethyl methacrylate polymers are normally used with eluents containing methanol, or mixtures of hexane and 2-propanol. The polymers are soluble in several common solvents (e.g. chlorinated hydrocarbons, tetrahydrofuran, etc.) resulting in poor column stability. This together with the fact that there are few indications that separations performed on these columns cannot be replicated on other phases, has resulted in declining use.

10.4.2.2 Supercritical Fluid Chromatography

The lower mobile phase viscosity and higher solute diffusivity in supercritical fluids often provide improved resolution and shorter separation times for the separation of enantiomers when compared with liquid chromatography. Rapid column equilibration after changes in the separation parameters in supercritical fluid chromatography shortens method development time. The ease of mobile phase removal makes supercritical fluid chromatography attractive for preparative-scale separations. The derivatized poly(saccharide) phases are stable under supercritical fluid chromatography conditions, even though the polymers are coated and not bonded to the silica support [28,39-43]. A large number of enantiomers have been separated on cellulose and amylose tris(3,5-

Table 10.6
General properties of glycopeptide chiral stationary phases

Property	Ristocetin A	Teicoplanin	Vancomycin
Molecular weight	2066	1877	1449
Stereogenic centers	38	23	18
Macrocycles	4	4	3
Sugar groups	6	3	2
Aromatic rings	7	7	5
Hydroxyl groups	21	15	9
Amide groups	6	7	7
Amine groups	2	1	2
pH stability range		3.8-6.5	4-7

dimethylphenyl carbamates) at 30°C with carbon dioxide-methanol containing 0.1% (v/v) triethylamine and 0.1% (v/v) trifluoroacetic acid mobile phases, usually with composition programming [117-121]. The glycopeptide stationary phases (section 10.4.3) were only slightly less successful than the poly(saccharide) derivatives and demonstrated a similar application range [117,122,123]. Other phases used successfully include immobilized cyclodextrins and chemically bonded low-mass synthetic selectors (Pirkle phases). Chiral stationary phases based on proteins, ligand exchange, and crown ethers are either unstable or ineffective in supercritical fluid chromatography.

Most enantiomer separations depend, at least in part, on multiple polar interactions between the enantiomers and chiral selector to form diastereomer complexes. These complexes are often too stable for the enantiomers to be eluted by carbon dioxide in the absence of polar modifiers. Optimum resolution is usually observed at low temperatures, frequently subcritical. These conditions typically result in increased peak separations accompanied by increased band broadening. The observed change in resolution depends on which factor is dominant. Mobile phase composition and temperature are the two most important parameters for optimizing resolution in the minimum separation time; pressure is often less important as the modified mobile phases are not very compressible.

10.4.3 Macrocyclic Glycopeptides (Antibiotics)

The macrocyclic glycopeptides are a relatively new class of chiral selector with favorable chromatographic properties and broad applicability [124-129]. So far, eight glycopeptides (vancomycin, teicoplanin, thiostrepton, rifamycin B, kanamycin, streptomycin, fradiomycin and ristocetin A) have been used for the separation of enantiomers by either liquid chromatography or capillary-electromigration separation techniques. Vancomycin, teicoplanin, and to a lesser extent ristocetin A, Table 10.6 and Figure 10.6, are generally used for liquid chromatography [130]. The macrocyclic glycopeptides can be bonded covalently to silica gel by multiple linkages involving the hydroxyl and amine groups of the glycopeptides, in such a way as to ensure their stability, while retaining their enantioselectivity [129]. All the glycopeptides contain an aglycone portion, made up of fused macrocyclic rings forming a characteristic “basket”

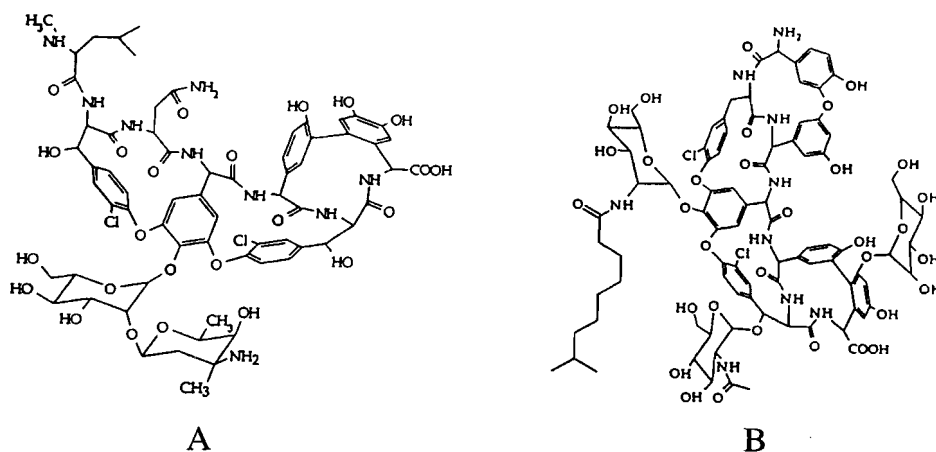


Figure 10.6. Structures of the macrocyclic glycopeptide chiral selectors vancomycin (A) and teicoplanin (B).

shape, with carbohydrate groups attached to the aglycone basket. In addition, each glycopeptide has a large number of stereogenic centers and various functional groups, promoting multiple interactions with different enantiomers. The application range of the macrocyclic glycopeptides is similar to protein-based stationary phases (section 10.4.4), with the advantage that the glycopeptide columns are more robust and efficient, as well as possessing a higher loading capacity.

The macrocyclic glycopeptides can be used in the normal-phase, reversed-phase, and polar organic solvent modes with few problems in changing from one mode to another. The enantioselectivity for each mode is generally different, increasing the versatility of the glycopeptide chiral selectors. If the compound has more than one functional group capable of interacting with the stationary phase, and at least one of those groups is on or near the stereogenic center, then the polar organic solvent mode should provide a good starting point. Methanol is the preferred solvent, with small amounts of acetic acid and triethylamine used to control retention and enantioselectivity. The total concentration of acid and base within the range 0.001 to 1 % (v/v) is used to control retention, and the ratio of acid to base, to control enantioselectivity. If the total concentration of acid and base required for elution exceed 1 % (v/v), then separation in the reversed-phase mode is indicated. If the total concentration of acid and base to achieve acceptable retention is less than 0.001 % (v/v), then separation in the normal-phase mode is indicated. Hexane-aliphatic alcohols (e.g. ethanol) are commonly used for normal-phase separations, and mixtures of organic solvent and buffer for the reversed-phase mode. The most suitable buffers for reversed-phase separations are ammonium nitrate, triethylamine-acetic acid, and sodium citrate. The pH range should be restricted to the stable operating range for the stationary phase, Table 10.6. Separations in the reversed-phase mode usually benefit from the presence of buffer (10–100 mM), even for neutral compounds. Enantioselectivity is usually highest in the reversed-phase mode if ionizable racemates

are separated in their neutral form. Tetrahydrofuran is the preferred organic solvent modifier for vancomycin, while for teicoplanin, methanol seems more versatile. Other factors affecting enantioselectivity include the buffer type, and ionic strength.

The excellent enantioselectivity of the macrocyclic glycopeptides is based on distinctive shape parameters related to multiple stereogenic centers, and to multiple binding sites that are readily available close to the stereogenic centers. Enantiomers are probably bound to the pocket of the glycopeptide by partial inclusion, particularly in the reversed-phase mode. Anionic or cationic sites on the glycopeptide can interact strongly with oppositely charged enantiomers. Carboxylic acids enter into strong electrostatic interactions with an ammonium center located in the aglycone basket of teicoplanin [126]. Close to this site are located a hydrophobic cavity, as well as additional hydrogen bonding and dipolar groups associated with the aglycone peptide bonds, and the pendent carbohydrate groups. These features provide a facile method for the separation of amino acid and peptide enantiomers. Hydrogen-bonding, dipole-type, and π - π interactions with the aromatic groups of the peptide side chains are favored in the normal-phase mode, and are also likely to contribute to a lesser extent to enantioselectivity in the reversed-phase mode. The availability of a large number of enantioselective binding sites provides the basis for broad applicability, but complicates the rational understanding of the chiral recognition mechanism, and the ability to predict absolute configurations from elution order. Given the large number of macrocyclic glycopeptides available for study, further examples of this class of chiral selector are likely to become available in the future.

10.4.4 Proteins

Proteins are composed of chiral monomers, organized in different sequences, providing a huge structural diversity. Specific folding (secondary and tertiary structures) and post-translational modification (e.g. glycosidation and sialinic acid modification) add to this diversity, creating chiral selectors with numerous binding sites of different stereoselectivity. Protein-based stationary phases are considered indispensable tools for analyzing stereoselective phenomena of biological significance (e.g. drug-protein binding studies), which are not discussed here. Their general use for analysis is in decline, however, since more rugged and efficient chiral stationary phases with a higher sample capacity are now available and suitable for the separation of enantiomers formally only possible on protein phases. The low sample capacity of protein phases can be a problem, even for analytical applications, and precludes their use for preparative-scale separations. The complexity of protein structures results in a limited mechanistic understanding of the separation process.

Typical proteins used for separating enantiomer include albumins (bovine and human serum), glycoproteins (α_1 -acid glycoprotein, ovoglycoprotein, avidin), and enzymes (cellobiohydrolase I, pepsin), Table 10.7 [28-32,131-134]. Proteins can be physically adsorbed on a suitable support for chromatography, but are usually prepared by covalently bonding the protein to a suitable support. Proteins can be bound to wide pore,

Table 10.7

Protein-based stationary phases for liquid chromatography

Protein	Molecular mass (kDa)	Carbohydrate content (%)	Isoelectric point
<i>(i) Albumins</i>			
Bovine serum	66	0	4.7
Human serum	65	0	4.7
<i>(ii) Glycoproteins</i>			
α_1 -Acid glycoprotein	41	45	2.7
Ovoglobulin	30	25	4.1
Avidin	66	7	10.0
<i>(iii) Enzymes</i>			
Cellobiohydrolase I	64	6	3.9
Pepsin	34.6	0	<1

chemically bonded silica gels (e.g. 3-aminopropylsiloxane-bonded silica) by coupling either the side-chain or terminal amino groups, or terminal carboxylic acid groups, to the chemically bonded support. The chiral recognition properties of adsorbed or bound proteins are often different to those of the protein in solution because of blocking of functional groups and/or conformational changes. To minimize denaturation, protein-bound phases are suitable for use with aqueous mobile phases in the pH range 3-8.

The α_1 -acid glycoprotein is built up of a single peptide chain containing 183 amino acids [132-134]. Five poly(saccharide) groups are linked to the peptide chain via the asparagine residues. The poly(saccharide) groups contain 14 sialic acid residues, giving the protein an acidic character with an isoelectric point of 2.7. At least two different stereogenic-binding sites have been identified for α_1 -acid glycoprotein. The main binding site is most likely a hydrophobic pocket, formed by an enrichment of hydrophobic amino acid residues, and containing numerous ionizable groups, both acidic and basic, allowing for the possibility of ionic binding of enantiomers. Separations are usually performed at pH 4-7, where the protein has a negative charge, and binds cationic enantiomers by electrostatic interactions. Neutral and acidic enantiomers are bound mainly by hydrophobic, hydrogen bonding, and dipole-type interactions. In addition, cationic enantiomers can be bound by adsorption of ion-pairs with the buffer as a source of counterions. Enantioselectivity generally depends on pH, type and concentration of organic modifier, buffer type and its ionic strength, and temperature. For cationic enantiomers, mobile phase pH is often the most critical parameter with enantioselectivity generally increasing at higher pH and retention with an increase in buffer concentration. For hydrophobic compounds, an organic modifier, such as propan-2-ol or acetonitrile, is required to optimize retention and enantioselectivity. The adsorption of organic solvent by the protein induces reversible changes in its conformation leading to changes in enantioselectivity in addition to those induced by competition with the enantiomers for adsorption at the binding site.

The α_1 -acid glycoprotein and ovoglobulin stationary phases have the broadest application range of the protein-based stationary phases. The albumins may provide improved resolution of acidic and zwitterionic enantiomers. Cellobiohydrolase I is

sometimes preferred for the separation of basic compounds, but is usually less effective than α_1 -acid glycoprotein and the albumin stationary phases, for the separation of neutral compounds.

10.4.5 Low-Mass Synthetic Selectors

Low-mass synthetic selectors are represented by a large number of chiral stationary phases developed from concepts of a rational model of the chiral recognition mechanism. They usually contain a single or small number of chiral centers near substituent groups that enter into attractive or steric interactions with the enantiomers. Separations are often rationalized using the three-point rule based on hydrogen bonding, π - π , and dipole-type interactions between complementary features of the chiral selector and enantiomers. The chiral selectors are relatively small and suitable for immobilization on silica gel or glass surfaces by standard chemical procedures. This results in efficient and durable phases with a comparatively high bonding density of the chiral selector. The chiral selectors are generally available in both single-enantiomer forms, facilitating reversal of the elution order for the quantification of the minor enantiomer in samples of high enantiomer purity.

10.4.5.1 Gas Chromatography

The development of amino acid ester, dipeptide, and diamide chiral stationary phases provided the first indication that enantiomers could be separated by gas chromatography (or any chromatographic technique) on stationary phases designed to promote multiple attractive interactions with an enantiomer [35,38,135]. Real interest and progress in chiral separations resulted from the preparation of diamide phases, grafted onto a poly(siloxane) backbone, suitable for coating on open tubular columns [136-138]. Stationary phases of this type can be prepared by a number of different methods, for example, from poly(cyanopropylmethyltrimethylsiloxanes) by hydrolysis of the cyano group and subsequent coupling of the carboxylic acid group with L-valine-*t*-butylamine or L-valine-1-phenylethylamide. Trifluoroethyl ester-functionalized poly(siloxanes) provide a suitable substrate for the introduction of a variety of selectors (e.g. L-valine-*t*-butylamide, L-1-naphthylethylamide, L-*t*-leucine-*t*-butylamide) by nucleophilic displacement. Methods have also been developed for immobilization of the poly(siloxane) stationary phases at relatively low temperatures to minimize racemization of the chiral selector. Separation of each chiral center by several dimethylsiloxane or methylphenylsiloxane monomers (about 7) seems essential for optimum resolution of enantiomers and good thermal stability [139]. The only commercially available example of this class of chiral selector is the poly(methylsiloxane) phase containing L-valine-*t*-butylamide (Chirasil-Val), which has been used to separate a wide range of racemic amino acid derivatives, amino alcohols, amines, hydroxy ketones, 2-hydroxy acids and their esters, 3-hydroxy acids, lactones, and sulfoxides [140,141]. It permitted for the first time, a single separation of all the enantiomers of the common protein amino acids after conversion to *N*, *O*, *S*-pentafluoropropanoate isopropyl ester derivatives, Figure 10.7

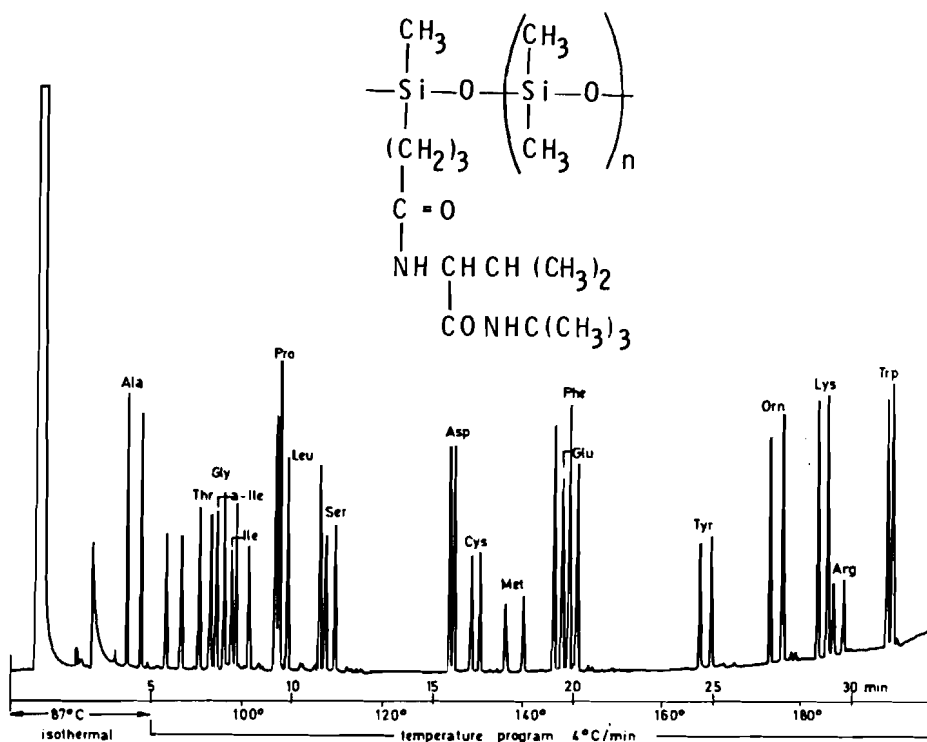


Figure 10.7. Separation of the enantiomers of the common protein amino acids by gas chromatography after derivatization on a 20 m x 0.25 mm I. D. open tubular column coated with Chirasil-Val. (From Ref. [142]; ©Elsevier).

[9,12,35,140-143]. This led to the development of the enantiomer labeling technique for quantitative amino acid analysis. The common protein amino acids are also fully separated on the cyclodextrin stationary phase (Chirasil- γ -Dex) [143]. The L-valine-1-phenylethylamide-containing stationary phase provides a facile method for the determination of the configuration of monosaccharides, as well as resolving enantiomers with neutral oxygen-containing functional groups [140]. Enantiomer separations by hydrogen bonding chiral stationary phases, such as Chirasil-Val, generally requires derivatization of the analyte in order to increase volatility and/or introduce suitable functional groups for additional hydrogen bonding association.

10.4.5.2 Liquid Chromatography

Following the pioneering work of Pirkle, a large number of chiral stationary phases based on low-mass synthetic selectors covalently bonded to silica gel for liquid chromatography have been described [21,144-148]. These phases have undergone evolutionary improvements or replacement to the extent that the number of stationary

Table 10.8

Covalently bonded synthetic low-molecular mass chiral selector stationary phases for liquid chromatography

Chiral selector	Column type
<i>(i) π-electron acceptor stationary phases</i>	
Dimethyl- <i>N</i> -3,5-dinitrobenzoyl- α -amino-2,2-dimethyl-4-pentenylphosphonate	α -Burke 2
3,5-Dinitrobenzoyl-3-amino-3-phenyl-2-(1,1-dimethylethyl)propanoate	β -GEM 1
<i>N</i> -(3,5-Dinitrobenzoyl)leucine amide	
<i>N</i> -(3,5-Dinitrobenzoyl)phenylglycine amide	
<i>N</i> -(3,5-Dinitrobenzoyl)tyrosine butylamide	ChyRoSine A
<i>N</i> -(3,5-Dinitrophenyl)valine urea	CHIREX 3010
<i>(ii) π-electron donor stationary phases</i>	
<i>N</i> -(1-Naphthyl)leucine ester	
{ <i>N</i> -[(1-Naphthyl)ethyl]amido}valine amide	CHIREX 3014
{ <i>N</i> -[(1-Naphthyl)ethyl]amido}indoline-2-carboxylic acid amide	CHIREX 3022
<i>(iii) π-electron acceptor/π-electron donor stationary phases</i>	
1-(3,5-Dinitrobenzamido)-1,2,3,4-tetrahydrophenanthrene	Whelk-O 1
<i>N</i> -(3,5-Dinitrobenzoyl)-1,2-diphenyl-1,2-diaminoethane	ULMO
<i>N</i> -(3,5-Dinitrobenzoyl)-1,2-diaminocyclohexane	DACH DNB
<i>N</i> -(3,5-Dinitrobenzoyl)-(1-naphthyl)glycine amide	CHIREX 3005

phases in current use is only a small fraction of those described. These surviving phases tend to have a broader application base than those that went before them, and form the basis of this discussion.

Some examples of the chiral stationary phases belonging to this group are summarized in Table 10.8 and Figure 10.8. The Pirkle-concept phases are designed to operate using attractive hydrogen bonding, π - π interactions, and dipole type interactions between the chiral selector and the enantiomers. If need be, analytes lacking the complementary functional groups for attractive interactions with the chiral selector can be derivatized to enhance their interactions with the chiral selector. Compounds containing amine or carboxylic acid groups close to the chiral center are usually derivatized first. Amines are converted to amides or carbamates and carboxylic acids to amides, anilides, or esters. The most commonly used derivatizing reagents contain either a naphthyl moiety or a 3,5-dinitrobenzoyl or 3,5-dinitrophenyl moiety to ensure maximum π - π interactions between the analyte and the chiral selector.

The chiral selectors belonging to the Pirkle-concept stationary phases possess either a strong electron-deficient aromatic group (π -acid) and/or an electron-rich aromatic moiety (π -base) for face-to-face and/or face-to-edge π - π interactions with complementary sites within the enantiomers [148]. In addition, these directing π - π interactions must be favorably supported by strong and directional hydrogen bonding and/or dipole-type interactions. These require hydrogen donor-acceptor groups (e.g. amide, carbamate, urea, sulfonamide, hydroxyl, etc.) readily accessible and close to the stereogenic center of the chiral selector. Bulky groups or rigid networks close to the stereogenic center of the chiral selector may enhance enantioselectivity by steric interactions. These stationary phases are primarily used in the normal-phase mode

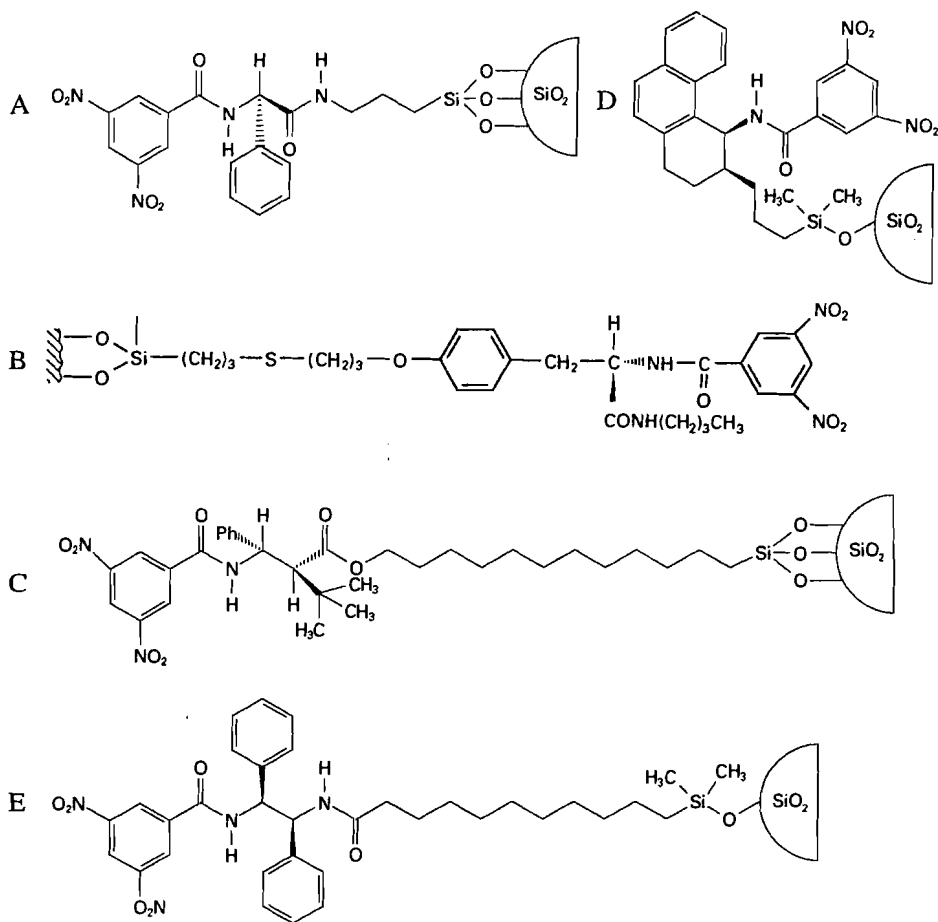


Figure 10.8. Synthetic low-mass chiral stationary phases. (A) DNP-phenylglycine; (B) ChyRoSine-A; (C) β -GEM 1; (D) Whelk-O 1; and (E) ULMO.

to enhance attractive interactions, and to allow the strength of these interactions to be optimized using competitive organic solvents in the mobile phase [149]. In most cases, enantioselectivity in the reversed-phase mode is lower than in the normal-phase mode because of the dominant solvation properties of water with respect to enantiomer binding interactions with the chiral selector [114].

Many of the chiral stationary phases have been developed by systematically applying the principle of reciprocity to the enantiomer binding interactions. A number of diverse racemates are analyzed on a chiral stationary phase containing as chiral selector the immobilized target molecule for the enantiomers of which a new selector is desired. The racemate showing the highest enantioselectivity in this system is selected, and

a chiral stationary phase prepared from one of its enantiomers. If the enantioselectivity is not adversely affected by the method of immobilizing the selector, then it is expected that a new stationary phase with superior properties for the separation of the target compound (and often-related compounds) will be obtained. To make the screening process more efficient combinatorial libraries of potential selectors can be used for target identification [150].

The latest generation of Pirkle-concept phases contains both π -acceptor and π -donor substituent close to the stereogenic center, Table 10.8. These phases have broad applicability to many compound classes, and are able to separate most enantiomers separated on the single π -acceptor or π -donor phases [29,144,151]. In the Whelk-O 1 phase, Figure 10.8, the π -acceptor and π -donor aromatic systems are held perpendicular to each other and form a flexible cleft in which the analyte is held by simultaneous face-to-face and face-to-edge interactions. Additionally, hydrogen bonding to the amide group and steric interactions occur near the stereogenic center. In general, racemates with an aromatic group and a hydrogen bond acceptor group near a chiral center, axis, or plane can be separated into enantiomers.

The synthetic low-mass chiral selectors, developed for liquid chromatography, are useful for supercritical fluid chromatography as well [28,39-43]. Only the Whelk-O chiral selector has been intentionally modified for use in packed column supercritical fluid chromatography by incorporating the selector as a side chain in a poly(dimethylsiloxane) matrix immobilized on silica gel (PolyWhelk-O) [152]. The PolyWhelk-O chiral stationary phase is more robust to high temperatures and mobile phase additives than its liquid chromatographic analog, and possesses similar enantioselectivity. Separations by supercritical fluid chromatography on synthetic low-mass chiral selectors generally follow those observed in normal-phase liquid chromatography, but are usually achieved in a shorter time, and with higher efficiency. In many cases, enantioselectivity can be enhanced using subcritical operating conditions. The scope of chiral separation methods is expanded by coupling columns of different enantioselectivity, facilitated by the low viscosity of supercritical fluids. An example is given in Figure 10.9, for the simultaneous separation of acidic and basic drugs on three coupled columns, each containing a different chiral stationary phase [122]. None of the individual coupled columns was able to separate all the enantiomers in the mixture.

10.5 CHIRAL MOBILE PHASE ADDITIVES

The chiral selectors used as mobile phase additives are generally the same as those employed for the synthesis of chiral stationary phases. Mobile phase additives offer greater flexibility in identifying suitable chiral selectors for a particular application. It is generally faster to screen additives than columns for a separation, and more economical to include a wider range of potential selectors in the screening process. Conventional stationary phases of higher efficiency than chiral stationary phases are used for the separation, improving the prospects for favorable resolution. The

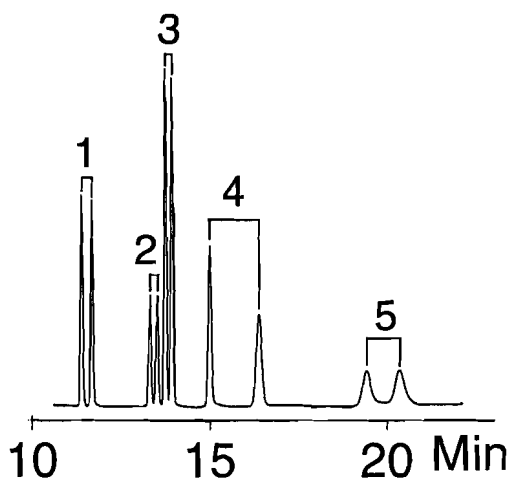


Figure 10.9. Separation of racemic drugs belonging to different structural types on series coupled Chiralpak AD, Chiralcel OD and Chirex 3022 columns with carbon dioxide-methanol (containing 0.5% triethylamine and 0.5% trifluoroacetic acid) as mobile phase using composition programming. Peaks: 1 = ibuprofen; 2 = fenoprofen; 3 = clenbuterol; 4 = propranolol; and 5 = lorazepam. (From Ref. [122]; ©Preston Publications).

main requirement for a mobile phase additive is that, the chiral selector should be soluble in suitable mobile phases, and be available as a single enantiomer or in high enantiomeric excess. The main limitation is that many chiral selectors interfere in the response of common chromatographic detectors, either causing sample detectability problems or poor baseline stability. This limits the use of some chiral selector and detector combinations, or necessitates the use of special techniques to separate the chiral selector and enantiomer before detection. This is easier to achieve in capillary electrophoresis, where chiral mobile phase additives represent the main approach for the separation of enantiomers, than for column liquid chromatography, where chiral mobile phase additives are used to a limited extent. The same problems exist for thin-layer chromatography, but in this case, the absence of a wide range of chiral stationary phases results in mobile phase additives being the commonly used approach for enantiomer separations.

10.5.1 Liquid Chromatography

The most important applications of chiral mobile phase additives in liquid chromatography are chiral ion-pair chromatography (section 4.3.3) and inclusion complex formation with cyclodextrins and similar chiral selectors. Other chiral mobile phase additives have been used only occasionally, and with modest success [1,3,32,153]. Multifunctional ion-pair reagents, such as 10-camphorsulfonic acid [154], derivatives of tartaric acid (e.g. di-n-butyltartrate) [155,156], peptides (e.g. N-carbobenzoxylglycine-L-proline) [157-161],

and cinchona alkaloids (e.g. quinine) [28] can form diastereomer ion pairs with racemic amines and carboxylic acids. For enantioselectivity, the counterion and enantiomer must enter into multipoint interactions where the stability of the ion-pair complex is different for each enantiomer. This requires the availability of additional hydrogen bonding, dipole-type, or steric interactions, close to the stereogenic center, to supplement the basic electrostatic interaction holding the ion-pair complex together. Suitable counterions, therefore, have an ionizable functional group close to their stereogenic center, additional binding sites in the vicinity of the stereogenic center, and a rigid structure to enhance stability differences in the ion-pair complexes. Ion-pair formation is favored in low-polarity solvents. Most applications of chiral ion-pair chromatography, therefore, employ organic solvents as the mobile phase with small amounts of an acid or base to promote sample ionization, and silica-based spacer bonded propanediol or porous graphitic carbon as stationary phases. The planar surface, high non-specific retention, and absence of functional groups, makes porous graphitic carbon (section 4.2.5) a particularly effective stationary phase for chiral ion-pair chromatography. The strong retention of the ion-pair reagent and/or their complexes allow more polar mobile phases to be used, including mobile phases containing water, as well as expanding the range of suitable chiral selectors that can be used as ion-pair reagents. It seems to facilitate separations by allowing greater flexibility in the choice of separation conditions and enhanced discrimination for the adsorption of diastereomer complexes.

Cyclodextrins and their derivatives are generally used as chiral mobile phase additives in the reversed-phase mode to promote inclusion complex formation, and to provide adequate solubility for the cyclodextrins. Native cyclodextrins and cyclodextrins with ionized functional groups (e.g. sulfated cyclodextrins) are thought to discriminate between enantiomers by differences in the stability of their inclusion complexes formed in the mobile phase [162-164]. Interactions between the cyclodextrin or enantiomer-cyclodextrin complex and typical (achiral) siloxane-bonded stationary phases are believed to be minimal. Alkylated cyclodextrins (e.g. methyl, hydroxypropyl), on the other hand, are believed to be strongly adsorbed by the stationary phase, with enantioselectivity resulting from the difference in stability of the diastereomer complexes formed with the adsorbed cyclodextrin [165]. In which case, enantioselectivity will depend on the concentration of the cyclodextrin in the mobile phase, the choice of achiral stationary phase (since this controls the non-selective retention of the enantiomers and surface coverage by the adsorbed cyclodextrin), temperature, and the type and concentration of organic modifier. Mixtures of cyclodextrin derivatives, with one cyclodextrin primarily forming complexes in the mobile phase and the second forming a dynamic stationary phase, can result in enhanced enantioselectivity in some instances [165]. This is likely to be the case, if one enantiomer binds more strongly with the cyclodextrin immobilized on the stationary phase, and the second enantiomer with the cyclodextrin with minimal interactions with the stationary phase. Buffers, if used at all, are used to suppress ionization of the analyte, or to improve peak shapes resulting from undesirable interactions with stationary phase silanol groups. Beyond the general control of retention, the role of organic modifier in enantiomer separations is not well defined. It is hypoth-

esized that the type of organic modifier can affect enantioselectivity by: (1) reducing the hydrophobic driving force for inclusion complex formation; (2) by destabilizing the analyte-cyclodextrin complex by enhancing dispersion interactions between the analyte and mobile phase; (3) by competing with the analyte for occupation of the cyclodextrin cavity; (4) by undergoing inclusion complex formation simultaneously with the analyte; and (5) by disturbing the stoichiometric equilibrium between water and the complex [166,167]. Chiral ion-pair reagents and cyclodextrin derivatives have also been used to separate enantiomers using packed column supercritical fluid chromatography [168,169].

10.5.2 Thin-Layer Chromatography

There are few chiral stationary phases available for the separation of enantiomers by thin-layer chromatography [58-60]. Layers prepared with microcrystalline cellulose and its acetate and benzoyl derivatives, are the most versatile and suitable for the separation of polar racemates with multiple hydrogen bonding and dipole interaction sites close to the stereogenic center [60,170-172]. They are used mainly in the reversed-phase mode with water-aliphatic alcohol mixtures as the mobile phase. The mobile phase composition affects enantioselectivity through competition with enantiomers for polar interactions at the binding sites, and by altering the conformation of the stationary phase due to solvent regulated swelling, which is believed to control inclusion complex formation. Several racemates have been separated on silica gel or silica-based spacer bonded propanediol layers impregnated with chiral selectors, such as D-galacturonic acid, N-carbobenzoxy-L-amino acids, 10-camphorsulfonic acid, etc., using low-polarity mobile phases [58,59]. Only octadecylsiloxane-bonded silica layers, impregnated with a copper (II) complex of N-(2-hydroxydodecyl)-4-hydroxyproline, and used in the reversed-phase mode for ligand-exchange chromatography, are used to any significant extent (section 10.6.2).

Chiral mobile phase additives provide a more versatile and cost-effective approach for enantiomer separations in thin-layer chromatography. Typically, chemically bonded layers with cyclodextrin and its derivatives, bovine serum albumin, or macrocyclic glycopeptides are used as chiral additives in the reversed-phase mode [59,60,172-178]. For β - and γ -cyclodextrins and their derivatives, a 0.1 to 0.5 M aqueous methanol or acetonitrile solution of the chiral selector is used as the mobile phase. Bovine serum albumin is generally used at concentrations of 1-8 % (w/v) in an aqueous acetate buffer of pH 5 to 7 or in a 0.5 M acetic acid solution, in either case with from 3-40 % (v/v) propan-2-ol (or another aliphatic alcohol), added to control retention. Enantioselectivity usually increases with an increase in concentration of the chiral selector, and may be non existent at low concentrations of the chiral selector.

The low efficiency and short migration distances typical of thin-layer chromatography limit useful separations to those with relatively large enantioselectivity factors. Absorption by the chiral selector can cause baseline instability and reduced sample detectability for quantitative measurements by scanning densitometry. The chiral sepa-

ration mechanisms employed in thin-layer chromatography are not specific to this technique, and any separation achieved by thin-layer chromatography, is also possible by column liquid chromatography.

10.5.3 Capillary Electrophoresis

The general use of capillary-electromigration separation techniques for enantiomer separations has grown significantly in the last few years [51-56]. The low-mobile phase consumption facilitates the economic use of even expensive chiral mobile phase additives. This together with the high separation efficiency of capillary-electromigration separation techniques enables successful conditions to be found for the separation of most enantiomeric mixtures. Capillary electrophoresis remains the dominant technique for enantiomer separations, but the frequency of use of capillary electrochromatography is increasing.

At equilibrium, the effective mobility of an enantiomer in the presence of a chiral selector in capillary electrophoresis is the sum of the electrophoretic mobility of each species containing the enantiomer, weighted by the mole fraction of each species. Assuming for a given separation the enantiomers exist as either the free enantiomer with a mobility, μ_R , or the enantiomer-chiral selector complex, $\mu_{C,R}$, then the effective mobility for the enantiomer is given by

$$\mu_{\text{eff},R} = (\mu_R + K_R[\text{CS}]\mu_{C,R}) / (1 + K_R[\text{CS}]) \quad (10.1)$$

where K_R is the formation constant for the enantiomer-chiral selector complex and $[\text{CS}]$ the chiral selector concentration. The equation has been written for the R configuration of the enantiomer (an identical equation can be written for the S configuration). The general requirement for a separation of enantiomers by capillary electrophoresis is that $\mu_{\text{eff},R} \neq \mu_{\text{eff},S}$. This requirement is met, if the complex formation constants or the mobility of the enantiomer-chiral selector complexes, are not identical ($K_R \neq K_S$ or $\mu_{C,R} \neq \mu_{C,S}$). When it can be reasonably assumed that differences in the enantiomer-chiral selector complex mobility are small (i.e. $\mu_{C,R} \approx \mu_{C,S} = \mu_C$) the enantioselectivity, $\Delta\mu_{\text{eff},R,S}$, is expressed by

$$\mu_{\text{eff},R,S} = \{(\mu_0 - \mu_C)(K_R - K_S)[\text{CS}]\} / \{1 + [\text{CS}](K_R + K_S) + K_R K_S [\text{CS}]^2\} \quad (10.2)$$

where μ_0 is the mobility of the analyte in the absence of the chiral selector. To maximize the enantioselectivity, a large difference in mobility between the analyte and the analyte-chiral selector complex is desirable (i.e. the vector terms μ_0 and μ_C should have opposite signs corresponding to migration of the free and complexed form of the analyte in opposite directions). The enantioselectivity is enhanced by a significant difference in the affinity of each enantiomer for the chiral selector (choose a chiral selector that maximizes the difference between K_R and K_S). In addition, for any chosen chiral selector, there is an optimum concentration of the chiral selector for maximum

enantioselectivity ($[CS]_{\text{opt}} = 1 / \sqrt{K_R K_S}$), which is unique for each analyte. Thus, when screening several racemates it is unlikely there will be a single chiral selector concentration that is optimum for the separation of all enantiomers. The analyte as well as the chiral selector may be neutral, anionic, cationic, or zwitterionic. In addition, buffer additives (other than the chiral selector) and the nature of the capillary wall (coated or uncoated), which may both affect the electroosmotic flow, the charge of the analyte, and the chiral selector determine the enantioselectivity and migration time in capillary electrophoresis. Several models have been proposed to describe the influence of the chiral selector concentration, pH, electroosmotic flow, buffer type, and charge type for the chiral selector and enantiomer, multiple binding equilibria, and organic solvent modifier on enantioselectivity determined as a difference in mobility for the R- and S-enantiomers [49-52]. As well as these mobility difference models, Vigh and co-workers introduced the chiral charged resolving agent migration model (CHARM) to optimize separation conditions for ionized chiral selectors, where the enantioselectivity is expressed as the ratio of the effective mobility of the R- and S-enantiomers [178,179]. All these models are complex, but provide reasonable agreement with experimental data when the full range of experimental parameters is known. They are most useful for simulating the influence of experimental parameters on enantioselectivity and migration time.

The above models provide an explanation for the observed reversal of the enantiomer migration order with the same chiral selector when different experimental conditions are employed [49,180]. The migration order is determined by the interaction of several parameters. Variation of these parameters is an effective means of changing the migration order so that a desired enantiomer (e.g. a minor component) is migrated before the opposite enantiomer of higher concentration. The enantiomer migration order is affected by: (1) the enantiomer-chiral selector formation constants; (2) the direction and magnitude of the free analyte mobility; (3) the direction and magnitude of the chiral selector mobility; (4) the direction and magnitude of the enantiomer-chiral selector complex mobility; (5) the direction and magnitude of the electroosmotic flow; and (6) the concentration of the chiral selector. These parameters, in turn, can be modified by subsets of experimental parameters providing a flexible, if some times confusing, number of options. Most mobile phase chiral additives for capillary electrophoresis are available only in a single-enantiomer form. In which case, change of the enantiomer migration order cannot be accomplished by a change of the configuration of the chiral selector.

For general use in capillary electrophoresis, a mobile phase chiral additive should possess the following properties: (1) a high degree of enantioselectivity for a wide range of analyte structures; (2) solubility and stability in all common electrolyte solutions; (3) transparency in the low wavelength UV region; (4) favorable kinetics for the enantiomer-chiral selector binding process to minimize additional band broadening; (5) enantioselectivity independent of pH and other buffer parameters; and (6) availability in high purity at a reasonable cost. These properties are reasonably represented by the cyclodextrins and their neutral and ionic derivatives, which account for about two thirds

Table 10.9

Charged cyclodextrin chiral mobile phase additives for capillary electrophoresis

Charged cyclodextrin	Number of ionic groups
α -, β -, γ -Cyclodextrin phosphate	6
Carboxymethyl β -, γ -cyclodextrin	3.2, 3.5
Carboxyethyl β -cyclodextrin	6
Succinyl β -cyclodextrin	3.5
Sulfobutyl ether β -cyclodextrin	3.5
Sulfated β -cyclodextrin	7-11
Sulfated γ -cyclodextrin	
Hepta-6-sulfato- β -cyclodextrin	7
Heptakis(2,3-dimethyl-6-sulfato)- β -cyclodextrin	7
Heptakis(2- <i>O</i> -methyl-3,6-di- <i>O</i> -sulfato) - β -cyclodextrin	14
Octakis(2,3-diacetyl-6-sulfato)- γ -cyclodextrin	8
Aminomethyl- β -cyclodextrin	1-2
Heptakis(6-methoxyethylamine-6-deoxy)- β -cyclodextrin	7
2-Hydroxypropyltrimethylammonium- β -cyclodextrin	
Mono-(6- δ -glutamylamino-6-deoxy)- β -cyclodextrin	
Heptakis(2- <i>N,N</i> -dimethylcarbamoyl)- β -cyclodextrin	

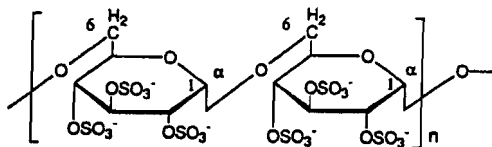
of all published enantiomer separations by capillary electrophoresis [53-56,63,66,181-184]. Other chiral mobile phase additives include chiral surfactants [55,185-187], proteins [188,189], macrocyclic glycopeptides [190], poly(saccharides) [191,192], and metal chelates (section 10.6.2). Nonaqueous capillary electrophoresis (section 8.2.6) has been explored to a limited extent for the separation of enantiomers with low water solubility or stability, and to modify the makeup of enantiomer-chiral selector interactions at the binding site compared with those in aqueous solution [193-195].

Cyclodextrins and their neutral derivatives, particularly β -cyclodextrin and its alkyl and hydroxyalkyl derivatives, are widely used for the separation of enantiomers in capillary electrophoresis [22,51,53,55,66,181]. The low solubility of β -cyclodextrin, in particular, limits separation possibilities for enantiomers that interact weakly with the cyclodextrin, because the optimum cyclodextrin concentration may be beyond the solubility limit. The solubility of β -cyclodextrin in aqueous electrolytes is less than 20 mM, while typical optimum concentrations of cyclodextrins for the separation of enantiomers span the range 1-100 mM (usually < 50 mM). The solubility of the derivatized β -cyclodextrins is about an order of magnitude higher than for the parent cyclodextrin, allowing the full concentration range for optimum enantioselectivity to be explored. Charged cyclodextrins, Table 10.9, are required for the separation of neutral enantiomers, and are also beneficial for ionized enantiomers, especially those of opposite charge to the cyclodextrin. For ionized enantiomers, this provides a means of maximizing the difference in electrophoretic mobility between the free and complexed forms of the enantiomer. General reactions used for the preparation of charged cyclodextrins produce randomly substituted products containing many different species that vary both in degree and in position of substitution. The range of

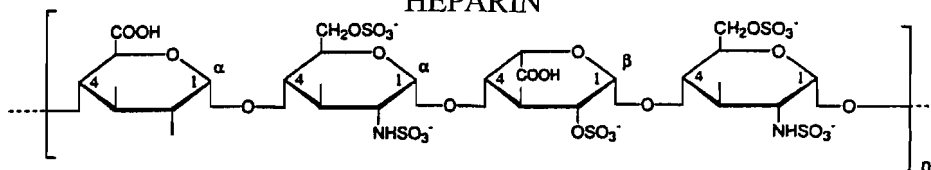
formation constants for the different enantiomer-cyclodextrin complexes may enhance the enantioselectivity. On the other hand, different degrees of substitution may be responsible for peak tailing, because of the formation of several inclusion complexes, and electromigration dispersion phenomena (section 8.2.5.1). Furthermore, variation in the number of ionic substituents affects the optimum chiral selector concentration, as well as ionic strength of the electrolyte solution. To obtain more rugged separation conditions, a number of single isomer cyclodextrins, which are fully sulfated on the primary hydroxyl groups and uniformly substituted on the secondary hydroxyl groups, have been developed, Table 10.9 [55,183,195-198]. Cyclodextrins with strong electrolyte functional groups are generally preferred, because then the pH of the electrolyte solution can be adjusted freely according to the needs of the analyte, without effecting the charge of the cyclodextrin. To increase enantiomer mobility differences, several parameters, such as type, number of substituents and cyclodextrin concentration, pH, ionic strength, composition of the electrolyte solution, and temperature must be carefully optimized [29,63,199,200]. Basic compounds are usually separated at acid pH, where enantiomers are positively charged, and the electroosmotic flow is minimized. High pH is more appropriate for the separation of acids. There are many examples, where using a cyclodextrin together with another mobile phase additive enhances the separation of enantiomers [51,53,56,201-203]. The second additive need not be chiral (e.g. sodium dodecyl sulfate), or a second chiral selector, such as a chiral surfactant or another cyclodextrin, is used. In most cases a charged cyclodextrin is used in conjunction with a neutral cyclodextrin. Achiral selectors can be used with neutral cyclodextrins to create electrophoretic mobility differences, where neutral cyclodextrins are used with neutral analytes, or to improve enantioselectivity when either the analyte or the cyclodextrin carries a charge. Regardless of the enantioselectivity of complex formation, a neutral chiral selector cannot separate neutral enantiomers in the absence of a mobility difference between the free and complexed form of the analyte. The use of a second charged selector means that the important competition process is not that between the free and complexed forms of the analyte, but between different complexed forms. For neutral analytes the charged cyclodextrin shows no enantioselectivity itself, in some cases, but provides a mechanism whereby the enantioselectivity of the neutral cyclodextrin can be expressed. In other cases, with charged analytes, both cyclodextrins can contribute to enantioselectivity.

Neutral cyclodextrins are also used in micellar electrokinetic chromatography with achiral surfactants to modify their enantioselectivity, particularly for the separation of hydrophobic analytes [53,55,185-187]. Enantioselectivity in this case results from differences in the distribution of enantiomers between the micellar pseudostationary phase and the cyclodextrin, as well as from the different migration velocities of the cyclodextrin and micelles. Neutral enantiomers can be separated based on differences in their equilibrium constants between the electrolyte solution and a charged chiral surfactant micellar phase, if the micelle has a different electrophoretic mobility to the free enantiomers. Suitable chiral surfactants include the bile salts (section 8.3.3), long alkyl-chain amino acid derivatives (e.g. sodium N-dodecanoyl-

DEXTRAN SULFATE



HEPARIN



CHONDROITIN SULFATE A

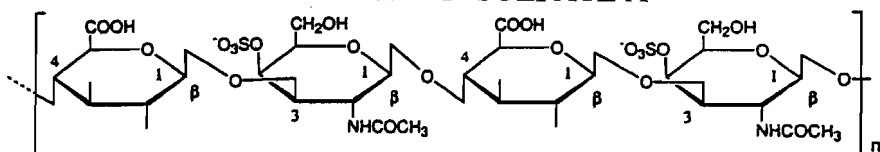


Figure 10.10. Poly(saccharide) chiral mobile phase additives for capillary electrophoresis. Heparin (molecular mass 7,000-20,000 with 2-3 sulfate groups per disaccharide), Chondroitin sulfate A (molecular mass 30,000-50,000 with 0.2-0.3 sulfate groups per disaccharide) and Dextran sulfate (molecular mass 7,300 with 6 sulfate groups per disaccharide).

L-valinate, N-dodecoxycarbonylvaline), polymer surfactants (e.g. poly[sodium N-undecenyl-L-valinate]), and n-alkyl- β -glucopyranosides. An important property of the glycosidic surfactants is that they can be charged *in situ* through complexation with borate anions, which allows the surfactant charge density to be adjusted to optimize enantioselectivity [204].

Many ionic poly(saccharides), such as heparin, chondroitin sulfates, dextran sulfate, and natural poly(saccharides), such as dextran, dextrin, pullulan, and their charged derivatives have been used as mobile phase additives for the separation of different enantiomers, Figure 10.10 [191,192,205,206]. Dextrins were found to have a wide application range, thought to be due in part to their helical structures. Enantiomer-chiral selector complexes seem to be weaker than for cyclodextrins, and it has not been demonstrated that enantiomer separations obtained by the poly(saccharide) chiral selectors cannot be obtained using cyclodextrins. Natural poly(saccharides) are typically complex mixtures of homologues and isomers, with a composition that can vary for different sources, resulting in differences in enantioselectivity.

The same macrocyclic glycopeptides used as immobilized chiral selectors for liquid chromatography (section 10.4.3) are also suitable chiral mobile phase additives for capillary electrophoresis [129]. All the macrocyclic glycopeptides are unstable in aqueous organic solvent mixtures, particularly at pH <4 or pH >9. This does not

generally prevent their use for capillary electrophoresis, but should be taken into account when selecting operating and storage conditions. It is believed that the amine groups of the glycopeptide are the primary sites for interaction. Higher enantioselectivity is generally exhibited for acidic compounds, or compounds containing an anionic group (e.g. carboxylic acid, phosphate, or sulfate group). There seems to be an enhancement in enantioselectivity when the acid group is either α - or β - to the stereogenic center. This is especially so for enantiomers which also contain a carbonyl group, an aromatic ring, or an amide nitrogen at the α -, β -, or γ -position to the stereogenic center. The choice of macrocyclic glycopeptide and its concentration, pH of the electrolyte solution, and concentration of organic modifier, are the primary parameters used to control enantioselectivity. The preferred organic modifier is propan-2-ol. In addition, proteins, such as serum albumins and α_1 -acid glycoprotein (section 10.4.4), are used as chiral mobile phase additives in capillary electrophoresis [188,189]. Depending on the pH, the proteins are either negatively or positively charged, and suitable for the separation of neutral or ionized enantiomers. A general feature of the use of macrocyclic glycopeptides and proteins as chiral mobile phase additives, is problems associated with adsorption of the chiral selector on the capillary wall (loss of resolution), and high background UV absorption (diminished sample detectability and poor baseline stability). A solution to these problems is provided by the partial-filling technique [53,207-210]. The buffer containing the chiral selector is used to fill the separation capillary up to a position below the detection region. The sample is introduced, and the capillary inserted into the electrolyte solution without the chiral additive, for the separation. The separation conditions are chosen such that the chiral selector does not migrate past the detector. In this way the enantiomers migrate through the separation zone and are then detected in a zone, which does not contain the chiral selector. Changing either the chiral selector concentration or the length of the separation zone is used to optimize separations.

10.6 COMPLEXATION CHROMATOGRAPHY

The rapid and reversible formation of charge-transfer complexes between metal ions and organic compounds that act as electron donors can be used to adjust selectivity in chromatography and electrophoresis [211]. Such coordinate interactions are influenced by subtle differences in the composition or stereochemistry of the donor ligand, owing to the sensitivity of the chemical bond towards electronic, steric, and strain effects. A number of challenging separations of stereoisomers and isotopomers has been achieved by complexation chromatography that would be difficult or impossible by other chromatographic methods.

10.6.1 Silver Ion Chromatography

Silver ion (or argentation) chromatography is based on the characteristic property of unsaturated organic compounds to form transient charge-transfer complexes with

Table 10.10

General considerations for predicting the elution order of unsaturated compounds in silver ion chromatography

-
- Unsaturated aliphatic and alicyclic compounds form more stable complexes than do aromatic compounds.
 - Methylene-interrupted dienes bind more strongly to silver ions than conjugated dienes, and monoenes bind more strongly than monoynes.
 - For methylene-interrupted dienes, the stability of complexes increases with the number of double bonds.
 - *cis*-Double bond isomers usually form more stable complexes than *trans*-double bond isomers.
 - Stability of complexes usually decreases with increasing chain length of the aliphatic substrate.
 - Stability of complexes usually decreases with increasing number of substituents attached to the double bond, and increases when deuterium or tritium replaces hydrogen.
-

transition metals in general, and with silver ions in particular [211-213]. Suitable electron donors are organic compounds containing π -electrons in various kinds of double and triple bonds, or containing heteroatoms (e.g. N, O or S) with electron lone pairs. For olefins, the silver ion is believed to act as an electron acceptor forming a σ -type bond between the occupied 2p orbitals of the olefinic double bond and empty 5s and 5p orbitals of the silver ion, and a π -acceptor backbond between the occupied 4d orbitals and the free antibonding 2p π^* orbitals of the olefinic double bond [213,214]. The stability of these complexes is sensitive to small changes in the bond electron density and steric factors affording separations of olefinic compounds according to the number, geometry, and position of the double bonds. Interactions of silver ions with the electron lone pairs of oxygen of the carbonyl group were implicated in the separation of triacylglycerols [214]. In general, conjugated dienes form weaker complexes with silver than isolated double bonds, and double bonds with substituents differing in size and position can frequently be separated based on differences in their complex stability constants, Table 10.10. The stability of the complexes increases at lower temperatures, and the low maximum temperature for useful separations effectively limits gas chromatography to the separation of volatile olefins [211].

Silver ion chromatography is used primarily in liquid [213,215-218], thin-layer [212,213,216,219], and supercritical fluid chromatography [220-222] for the separation of fatty acid derivatives and triacylglycerols, and to a lesser extent, terpenes, sterols, carotenoids, and pheromones. Silver ion chromatography is widely used on its own, or as a preliminary simplification step, to elucidate the structures of fats, oils and lipids [213]. It is possible to fractionate animal or fish oils into fractions with zero to six double bonds. Species with the same total number of double bonds can be separated based on the difference in the number of double bonds in individual acyl residues.

Silver-loaded layers for thin-layer chromatography are usually prepared by slurring silica gel with an aqueous solution of silver nitrate (1-2 % w/v), or by immersing precoated silica gel layers in a 0.5 % (w/v) solution of methanolic silver nitrate

for five minutes [212,213,219]. Columns for liquid chromatography are prepared by loading a solution of silver nitrate onto a prepacked silica gel column, or a silica-based, benzenesulfonic acid, cation-exchange column [212,213]. The silver-loaded, cation-exchange column is the preferred approach, and results in more stable retention and minimal contamination of collected fractions with silver salts. Separations are usually performed in the normal-phase mode with chlorinated hydrocarbon solvents containing small amounts of acetonitrile or ethyl acetate for the separation of fatty acids. Hexane containing small amounts of acetonitrile, ethyl acetate, acetone, or acetic acid is used for the separation of derivatized fatty acids and triacylglycerols [213,215,217,218]. Acetonitrile is a particularly effective solvent for eluting compounds containing several double bonds from silver-loaded, cation-exchange columns. Gradient elution is also commonly used for the fraction of complex mixtures. Reversed-phase liquid chromatography is usually the method of choice for analyzing molecular species from complex lipids (e.g. phosphatidylcholines) [213]. In this case, silver nitrate is used as a mobile phase additive. This results in a decrease in retention due to an increase in the hydrophilic character of the complex compared to the parent analyte. Varying the concentration of silver nitrate in the mobile phase enables the retention of the complexed species to be changed over a wide range. Since silver nitrate solutions are corrosive and stain clothing, this approach is not as popular as those based on silver-loaded, cation-exchange columns. So far, applications of silver-loaded packings in supercritical fluid chromatography have been limited to the analysis of lipids using silica-based, silver-loaded, cation-exchange sorbents, silver-complexed, dicyanobiphenyl-substituted poly(dimethylsiloxane) encapsulated sorbents, or silver-complexed 8-quinolinol bonded sorbents [42,220,221]. All phases provide a good separation of fatty acid methyl esters according to their degree of unsaturation, but the silver-loaded, cation-exchange sorbents require the use of organic solvent modifiers, restricting detector options to the UV absorption or evaporative light-scattering detectors. In general, separations by supercritical fluid chromatography show small selectivity differences compared with similar separations by normal-phase liquid chromatography. Both techniques are valuable for the separation of triacylglycerols.

10.6.2 Ligand-Exchange Chromatography

Ligand-exchange chromatography is used to resolve enantiomers of amino acids and their derivatives, and some α -hydroxycarboxylic acids, and compounds with an imide structure capable of forming bidentate complexes with transition metal ions, such as copper, zinc, and nickel [17,223-225]. Almost all practical examples employ copper (II) amino acid complexes as the chiral selector in liquid or thin-layer chromatography. Based on its high enantioselectivity, ligand-exchange chromatography was at one time the principal method for separating amino acid enantiomers. Relatively poor column efficiency and effective competition from other methods (e.g. cyclodextrin and macrocyclic glycopeptide stationary phases), however, has resulted in a marked decline in its use in recent years. A limited amount of work indicates that ligand-exchange

chromatography is suitable for the separation of amino acids and their derivatives by capillary electrophoresis [226]. Ligand-exchange chromatography is also the basis for the separation of oligosaccharides on metal-loaded, cation-exchangers (section 4.3.7).

There are three general approaches for the separation of enantiomers by ligand-exchange chromatography. The chiral selector, a Cu (II) amino acid complex, can be bonded to a silica support by a spacer arm connected to the amino acid at a position that does not interfere in the chelation mechanism [223,225,227]. The Cu (II) amino acid complex can be physically adsorbed on an octadecylsiloxane-bonded silica [225,228] or porous graphitic carbon [229,230] sorbent by attaching a hydrophobic group to a position on the amino acid that does not interfere in the chelation mechanism. Or, the copper (II) amino acid complex can be used as a mobile phase additive [223,225]. The highest enantioselectivity for amino acids and their derivatives is usually obtained using a single enantiomer of proline, 4-hydroxyproline, or histidine methyl ester as the chelating ligand. Separations are performed in the reversed-phase mode with the enantioselectivity optimized by varying the copper (II) concentration, pH, and the type and concentration of organic modifier.

In chiral ligand-exchange chromatography, there is no direct contact between the chiral selector and the analyte. Their interaction is mediated by a metal ion, which simultaneously binds the chiral selector and the enantiomers to be separated. Equilibration of the complex with an enantiomeric mixture capable of complexing with the metal, results in one of the previously chelated amino acids being displaced, forming a mixture of diastereomeric complexes. These complexes are separated based on the difference of stability constants for metal complexes forming part of the stationary phase, or adsorbed onto the stationary phase. Formation of diastereomeric complexes in the mobile phase results in a separation based on differences in the solubility of the complexes in the mobile phase and their adsorption by the stationary phase. Slow ligand-exchange processes are the primary cause of poor chromatographic efficiency.

10.6.3 Enantioselective Metal Complexation Gas Chromatography

Metal (II) bis[3-(trifluoroacetyl)-(1R)-camphorate] and bis[3-(heptafluorobutanoyl)-(1R)-camphorate] of nickel, cobalt, and manganese dissolved in a noncoordinating solvent, such as poly(dimethylsiloxane), or incorporated into the structure of a poly(dimethylsiloxane), Figure 10.4, have emerged as highly selective stationary phases for the separation of a variety of stereoisomers that includes hydrocarbons and oxygen, nitrogen, and sulfur-containing electron-donor analytes (e.g. cyclic ethers, aziridines, thiranes, ketones, and aliphatic alcohols) by gas chromatography [16,35,38]. These complexes are also suitable for the determination of the enantiomer composition of volatile insect pheromones. Although the camphorates are the most widely evaluated and the only metal terpeneketonate complexes available as immobilized stationary phases, a number of other terpeneketonates have been evaluated as complexing ligands, as well as some further metals [16]. Selectivity results from the difference in stability constants for the fast and reversible chemical equilibrium between the

metal coordination compound and analytes. Thermal instability of the coordination complexes, and small stability constants at higher temperatures, restrict separations to volatile compounds that can be separated at temperatures less than about 125°C. Many earlier applications of metal coordination compounds, can now be achieved using cyclodextrin derivatives (section 10.4.1.1). Supercritical fluid chromatography employing immobilized metal coordination compounds is suitable for the separation of thermally or configurationally labile analytes of lower volatility than required for gas chromatography [16,231].

10.7 SEPARATION OF ENANTIOMERS AS COVALENT DIASTEREOMER DERIVATIVES

Enantiomers containing a reactive functional group can be derivatized with a single-enantiomer reagent to produce a mixture of diastereomers [1,2,28,29,232-236]. By itself, this does not guarantee that a separation will be obtained, but it does make possible a separation in the absence of a chiral selector, using conventional separation systems. A useful separation depends on the difference in physical properties of the diastereomers, the extent to which these differences influence the relative distribution of the diastereomers between phases in the chromatographic system, and the separation efficiency of the chromatographic system. In contemporary practice, methods based on the formation of covalent diastereomer derivatives have continued to decline in popularity, as a wider range of options for direct separation by chiral chromatography and electrophoresis became available. However, formation of covalent diastereomer derivatives remains a viable option for many compounds, and sometimes it is the only option available.

Several factors need to be considered in the selection of reagents and reaction conditions for the formation of diastereomer derivatives. The analyte must contain at least one functional group capable of reaction with the derivatizing reagent. A wide range of reagents is available for reaction with amines, hydroxyl (alcohol and phenol groups), and carboxylic acids, with a smaller number for aldehydes, epoxides and thiols, Table 10.11. The most widely used reactions employ relatively mild conditions, and short reaction times, to minimize racemization of the chiral compounds. The diastereomer transition states should have similar conversion rates for the enantiomers (absence of kinetic resolution) and similar stability in the reaction medium. The derivatizing reagent must be available as a single enantiomer in high enantiomer purity, and have an acceptable shelf life. To detect the presence of 0.5% of a minor enantiomer in a mixture of enantiomers requires a reagent of at least 99.9% enantiomer purity. It is advantageous if the derivatizing reagent is available in both the R- and S-form, to allow elution order reversal, to facilitate the detection of minor enantiomer components. Derivatization with suitable reagents also provides a method to improve detection properties of the enantiomers. This applies particularly to UV absorption properties, or the introduction of a fluorophore for fluorescence detection. It should be noted, however, that the UV

Table 10.11

Reagents for the formation of covalent diastereomer derivatives

(i) Primary and secondary amines

N-Trifluoroacetylpropyl chloride

1-(4-Nitrophenylsulfonyl)propyl chloride

 α -Methoxy- α -(trifluoromethyl)phenylacetyl chloride2-Methyl-2 β -naphthyl-1,3-benzodioxole-4-carboxylic acid chloride

Menthyl chloroformate

1-(9-Fluorenyl)methyl chloroformate (Fmoc)

2-(6-Methoxy-2-naphthyl)-1-propyl chloroformate (NAP-C)

1-Phenylethylisocyanate

1-(1-Naphthylethyl) isocyanate

2-(6-Methoxy-2-naphthyl)ethyl isothiocyanate (NAP-IT)

1,3-Diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate (DANI)

(2,3,4,6-tetra-*O*-acetyl)- β -glucopyrasonyl isothiocyanate (GITC)4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N,N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-PyNCS)

N-[(2-isothiocyanato)cyclohexyl]pivalinoyl amide (PDITC)

1-Fluoro-2,4-dinitrophenyl-5-alaninamide (Marfey's reagent)

*(ii) Alcohols and Phenols**trans*-Chrysanthemoyl acid α -Methoxy- α -(trifluoromethyl)phenylacetic acid (Mosher's reagent) α -Methoxy- α -(trifluoromethyl)propionic acid (MTPA)2-*tert*-Butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid

2-Phenylpropionyl chloride

1-Phenylethylisocyanate

1-(1-Naphthylethyl) isocyanate

Menthyl chloroformate

1-(9-fluorenyl)ethyl chloroformate (FLEC)

(iii) Carboxylic acids

2-Butanol

3-Methyl-2-butanol

Menthyl alcohol

2-Octanol

1-Phenylethanol

 α -Methyl-4-nitrobenzylamine

1-(1-Anthryl)ethylamine

1-(4-Dansylaminophenyl)ethylamine

2-[4-(1-Aminoethyl)-phenyl]-6-methoxybenzoxazole

(iv) Thiols

N-[(2-isothiocyanato)-cyclohexyl]-pivalinoyl amide (PDITC)

4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N,N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-PyNCS)

and fluorescence properties of each diastereomer derivative are not necessarily identical, and should be checked by calibration. The separation of diastereomers is often enhanced when the stereogenic centers of the enantiomer and the reagent are in close proximity in the derivative, and when the reagent is comprised of conformationally immobile groups, or contains bulky groups, attached directly to the stereogenic center.

The most likely sources of error in establishing the enantiomer composition of a mixture are the presence of enantiomeric impurities in the derivatizing reagent,

racemization during the derivatization reaction, and different rates of reaction for individual enantiomers. In general, methods based on the formation of covalent diastereomer derivatives require more effort for validation than direct methods, owing to the larger number of factors that affect the results. If the reaction is to be used for preparative purposes, easy conversion of the diastereomers into the parent enantiomers is also a necessary condition.

Currently, several hundred reagents are available for the preparation of diastereomer derivatives, and this list continues to grow. In consequence, any type of comprehensive coverage is impossible here. As well as the nature of the reactive group, cost, reactivity, stability, and availability are important selection criteria, since in advance of trial experiments, success cannot be guaranteed for analytes not studied previously. For trace analysis, the choice of a more selective and sensitive detector for the analysis may minimize the number of options to reagents with suitable characteristic detector properties.

The nature of the reactive group defines the possible application range for each reagent. For amines activated carboxylic acids (e.g. acid chlorides), chloroformates (forming carbamate derivatives), isocyanates (forming urea derivatives), isothiocyanates (forming thiourea derivatives) are popular choices [234,237-247]. Marfey's reagent (Table 10.11) is widely used to derivatize peptides and for other applications. The nucleophilic attack of the amine group on the C-F bond activated by the two nitro groups on the aromatic ring results in a smooth reaction to form aniline derivatives with good UV detectability [248,249]. The naproxen reagents (NAP-C and NAP-IT) have strong chromophores, but weak fluorescence. A number of reagents indicated in Table 10.11 and suitable for fluorescence detection. The reaction of amino acids with o-phthalaldehyde and a single enantiomer thiol (e.g. N-butyrylcysteine) result in the formation of highly fluorescent isoindole derivatives [234]. α -Methoxy- α -(trifluoromethyl)phenacetyl chloride and α -methoxy- α -(trifluoromethyl)propionic acid form stable derivatives with a wide range of amines for gas and liquid chromatography [234,237].

For alcohols, activated carboxylic acids or acyl nitriles (forming ester derivatives), chloroformates (forming carbonate derivatives) and isocyanates (forming carbamate derivatives) are widely used [234,250,251]. Dicyclohexylcarbodiimide can be used as the coupling agent for carboxylic acid reagents, but *in situ* transformation of the acid to the acid chloride is more widespread. Acid chlorides, anhydrides, and acyl cyanides usually require strictly anhydrous conditions and a catalytic amount of an organic base (e.g. N,N-dimethylaminopyridine) for quantitative reaction. Because of the lower reactivity of hydroxyl groups with isocyanates and chloroformates, a catalyst (organic base) is usually also required.

The most frequently used approaches for derivatizing carboxylic acids are esterification with a variety of single-enantiomer alcohols, or formation of amides with single-enantiomer amines [234,252]. The formation of amide derivatives requires activation of the carboxylic acid by formation of the acid chloride with thionyl chloride, mixed anhydrides with chloroformates, N-acylimidazoles with 1,1-carbonyldiimidazole or N-acylureas with dicyclohexylcarbodiimide. Esterification reactions generally re-

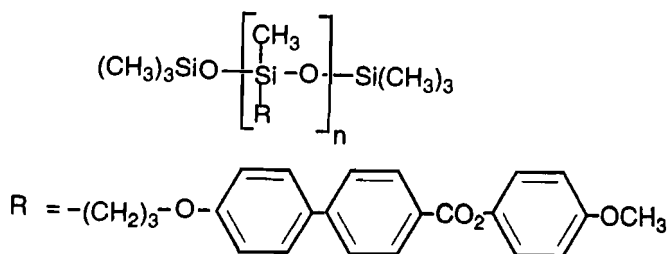
quire harsh conditions, and this should be considered if either the enantiomer or the diastereomer derivative is conformationally labile or unstable.

For liquid chromatography, both the reversed-phase and normal-phase mode is used to separate diastereomer derivatives. Normal-phase chromatography is particularly effective for separating diastereomers of moderate polarity in the absence of strong hydrogen bond acid/base functional groups. The requirements for supercritical fluid chromatography are similar to normal-phase liquid chromatography. For gas chromatography, the volatility and thermal stability of the derivatives is an important factor in selecting suitable reagents. The formation of diastereomer derivatives for enantiomer separations by capillary electrophoresis is not established as well as it is for chromatography [253-257]. Derivative formation in capillary electrophoresis has frequently been performed to improve sample detectability, especially in combination with laser-induced fluorescence detection. The use of chiral mobile phase additives is the favored approach for the separation of enantiomers in capillary electrophoresis (section 10.5.3).

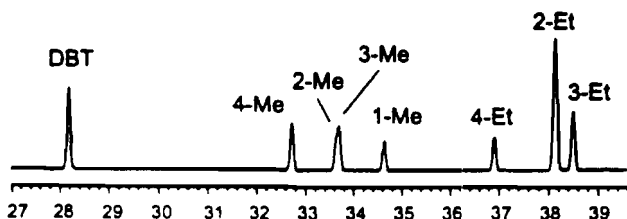
10.8 LIQUID-CRYSTALLINE STATIONARY PHASES

Liquid crystalline stationary phases are used for the separation of positional and geometric isomers of rigid molecules, such as benzene derivatives, terpenes, polycyclic aromatic compounds, polychlorinated biphenyls, and steroids [258-261]. Nearly all practical applications result from their use in gas chromatography, while rather limited success has been demonstrated for liquid and supercritical fluid chromatography. Liquid crystals exhibit the mechanical properties of a liquid while retaining some of the anisotropic properties of the solid state. This preservation of order, permits shape selectivity, while the liquid properties result in acceptable chromatographic efficiency. Several hundred liquid-crystalline phases of different chemical types have been used in gas chromatography. They all have in common a markedly elongated, rigid, rod-like structure, and generally have polar terminal groups. The most common types are Schiff bases, esters, azo, and azoxy compounds [258-263]. At their melting point, these compounds are transformed from a solid crystalline state to ordered smectic or nematic liquid states. The smectic configuration is more ordered than the nematic configuration, and occurs at a lower temperature for compounds that exhibit both phase transitions. At the clearing temperature, the liquid-crystalline state is transformed into an isotropic liquid. The temperature range bordered by the solid melting point and the clearing temperature defines the liquid-crystalline phase region. Supercooling coated liquid-crystalline phases allows some phases to be used below their solid melting point, if adequate column efficiency is maintained. The phase loading and support type can also alter the separation characteristics of liquid-crystalline phases. The thin film of liquid phase in immediate contact with the support has properties influenced by interactions with the support surface. As the film thickness builds up, these surface forces dissipate quickly.

Most monomeric liquid-crystalline phases provide limited opportunities for use in open tubular columns because of poor column efficiency, poor coating charac-



DB-5 MS



SB-Smectic

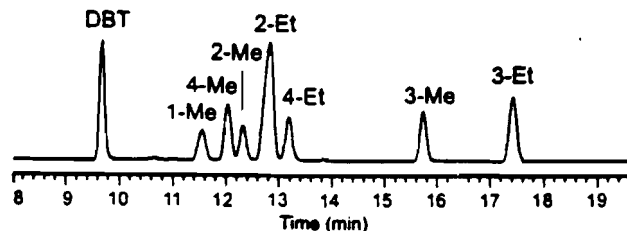


Figure 10.11. Structure of the SB-smectic side-chain liquid-crystalline poly(siloxane) stationary phase. The poly(methylsiloxane) backbone is substituted with about 50 % 3-[4'-(4-methoxyphenoxy-carboxyl)biphenyl-4-yloxy]propyl groups. Separation of dibenzothiophene (DBT) and four methyl and three ethyl isomers on a poly(dimethyldiphenylsiloxane) stationary phase containing 5 mol % phenyl (DB-5 MS) and the SB-smectic side-chain liquid-crystalline poly(siloxane) stationary phase. (From ref. [268]; ©Elsevier).

teristics, poor thermal stability, high column bleed, and limited temperature operating ranges. These shortcomings were largely overcome by the development of side-chain liquid-crystalline poly(siloxane) phases [264-267]. The biphenylcarboxylate ester poly(siloxane), Figure 10.11, is the most widely used liquid-crystalline stationary phase. It can be immobilized on open tubular columns, and has a useful temperature range from about 100 to 250°C isothermal (270°C temperature programmed). This phase has favorable properties for the separation of alkyl substituted, and ring heteroatom substituted, isomers of polycyclic aromatic compounds [268].

The general elution order of geometric and positional isomers of rigid molecules, on liquid-crystalline stationary phases in gas chromatography, correlates with their length-to-breadth ratio and planarity [268,269]. The length-to-breadth ratio and thickness are calculated as the dimensions of a box, drawn to enclose the atoms of the molecule. Retention normally increases with the length-to-breadth ratio with differences in planarity (thickness), vapor pressure, and polarity being important in some cases, possibly leading to inversion of the predicted order. Long and planar molecules fit better into the ordered structure of the liquid-crystalline phase, whereas nonlinear and nonplanar molecules do not permeate so easily between the liquid crystal molecules, and are less retained. The elution order of polycyclic aromatic compounds on the SB-smectic phase (Figure 10.11) is strongly correlated with the reversed-phase liquid chromatographic retention of the same compounds on polymeric octadecylsiloxane-bonded silica sorbents [269]. No single phase is likely to separate all components of a complex mixture of isomers. A liquid-crystalline phase is a useful component of a multicolumn strategy for gas chromatography, because of its complementary separation mechanism to phases that separate isomers based on vapor pressure or polarity differences.

Liquid-crystalline coated and chemically bonded phases have demonstrated limited shape selectivity in liquid chromatography [261,270-274] and modest success in supercritical fluid chromatography [261,275,276]. The low bonding density of siloxane-bonded phases probably contributes to the loss of long-distance order in the bonded phase structure. Shape-selectivity of these phases is generally similar to polymeric octadecylsiloxane-bonded phases (section 4.2.2.1) indicating that the contribution of the liquid-crystalline substituent is of limited importance. As for gas chromatography, side-chain polymeric liquid-crystalline stationary phases show greater retention of the liquid-crystalline order, and better prospects for shape-selective separations. Useful applications have still to be demonstrated for these phases, however.

10.9 REFERENCES

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Chapter 11

Laboratory-Scale Preparative Chromatography

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11.1 INTRODUCTION

The purpose of preparative-scale chromatography is the isolation of compounds with a specified purity, in amounts suitable for their intended application [1-6]. This might include the isolation of materials for structural elucidation, for biological or sensory evaluation, for use as analytical standards, for organic synthesis, or for commerce. The scale of the process varies considerably, and includes laboratory, pilot plant, and process-scale operations. For structural elucidation and initial biological activity screening, 30-50 mg of pure substance is usually adequate; for use as an analytical standard, > 100 mg is required; for synthesis, gram quantities will likely be needed; while, at the production scale, kilograms and upwards are possibly desirable. For the first three applications, separations can be handled in the laboratory, while for pilot plant and process-scale separations, special purpose equipment and facilities are usually required. In addition, process-scale separations must be performed to satisfy economic factors, often of less significance for laboratory-scale separations. For these reasons, process-scale separations are not treated explicitly in this chapter. Process-scale chromatography is of considerable importance for the manufacture of high-value added products, such as pharmaceuticals, flavors and fragrances, and biotechnology products, where the rather high unit costs of purification are not considered prohibitive [2,6-11].

The general layout of this chapter is to proceed from simple to more sophisticated techniques based on liquid chromatography, and then discusses other separation approaches. Liquid chromatography is the laboratory-scale technique of choice for the isolation and purification of materials that cannot be handled by crystallization or simple distillation. An exception is thermally stable and volatile mixtures, for which gas chromatography is the preferred method. The advantages of other methods are indicated at the point they are introduced.

11.2 THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography (TLC) is a simple and undemanding technique, suitable for the separation and isolation of compounds of low volatility in amounts up to about 1 g [4,6,12,13]. Micropreparative separations (\approx 2-10 mg) are possible at higher resolution on high-performance layers. Limited zone capacity restricts the number of components that can be separated to about 2-5 using conventional development techniques. Scale up to larger sample loads is achieved by increasing the thickness of the layer, and the length of the plate edge along which the sample is applied. The sample capacity increases roughly with the square root of the layer thickness, but resolution is generally less for thicker layers. Laboratory-made preparative plates, in sizes up to 20 x 100 cm with sorbent layers up to 1 cm thick are possible, but these dimensions are exceptional. More typical are 20 x 20 or 20 x 40 cm plates with sorbent layers 0.5 to 2 mm thick. Layers of these dimensions, prepared from silica gel, alumina or chemically bonded sorbents with an average particle size of about 25 μ m, are commercially available.

Their broad particle size range, however, is one factor that contributes to their low efficiency. Tapered layers, prepared with a gradual increase in layer thickness from 0.3 mm at the bottom to 1.7 mm at the top, represent one approach to improving resolution [14]. The tapered layer results in the formation of a negative velocity gradient in the direction of mobile phase migration. As a result, the lower portion of a zone moves faster than the upper portion, keeping each component focused as a narrow band. Multiple development techniques on conventional layers provide another approach to improve separation performance (section 6.6.3). Conventional and tapered layers with concentrating zones are available for convenient sample application.

Sample application is a critical step in preparative thin-layer chromatography [14, 15]. The sample, usually as a 5-10% (w/v) solution in a volatile organic solvent, is applied as a band along one edge of the layer. The maximum sample load for a silica layer 1.0 mm thick is about 5 mg / cm² (lower for cellulose and reversed-phase layers). Any of the automated band applicators for analytical thin-layer chromatography are equally suitable for preparative-scale thin-layer chromatography, and are the method of choice for sample application (section 6.5). A margin of 1-2 cm is frequently left at either vertical edge of the layer to minimize uneven development. Manual sample application by syringe or pipette should be performed carefully, to avoid damaging the separation layer, resulting in irregularly shaped zones after development. Sample zones can be focused to some extent by a short development (≈ 1 cm) with a strong solvent. Some of the problems with manual sample application can be circumvented using layers with a concentrating zone. The concentrating zone is a narrow strip of inert silica of low retention that butts up to the separation layer. The sample solution can be applied to the concentrating zone without much skill. The soluble sample components are migrated to the boundary between the concentrating zone and separation layer with a strong solvent, where they are focused into a narrow band.

Preparative-scale plates are usually developed in rectangular glass tanks (e.g. 21 x 21 x 9 cm) lined with thick filter paper on all sides. The chamber is charged with sufficient mobile phase for the development step, and to soak the filter paper liner. Equilibration of the vapor phase typically requires 1-2 h. Saturated developing chambers are preferred to minimize the formation of irregular solvent fronts and developed sample bands. The plates are usually inserted in a rack that holds them in a vertical position, and allows several plates to be developed simultaneously. Ascending development typically requires 1-2 h for a solvent-front migration distance of 18 cm.

Most layers for preparative-scale thin-layer chromatography contain a fluorescent indicator, which simplifies locating separated UV absorbing bands. Non-UV absorbing compounds can be located by physical or chemical techniques. Spraying with water, or exposing the layer to iodine vapor, provide a reversible indication of zone positions. Spraying one edge of the layer with a chemical reagent (a small vertical section is usually detached for this purpose), or using detectable reference compounds separated along with the sample, provide alternative approaches for locating sample zones of interest. Separated zones are marked for removal, and scraped off the plate with a spatula or similar tool. A number of devices based on the vacuum suction principal

Table 11.1

Comparison of separation techniques for preparative thin-layer chromatography

Parameter	Method of mobile phase migration		
	Capillary flow	Forced flow	Centrifugal
Layer thickness (mm)	0.5-2	0.5-2	1-4
Separation length (cm)	18	18	12
Separation mode	Linear	Linear	Circular
Isolation method	Off-line	On-line	On-line
Typical sample size (mg)	50-150	50-300	50-500
Number of separated bands	2-5	2-7	2-12

are available for removing marked zones from the layer. These devices work like a vacuum cleaner, and collect the desired sample bands in a Soxhlet thimble or a glass chamber with a fritted base. The sample is separated from the adsorbent by solvent extraction or elution. Ethanol (but not methanol due to the relatively high solubility of silica in methanol) or acetone, are commonly used for direct extraction. In some cases, addition of a small quantity of water to dampen the silica gel, before shaking for several minutes with several portions of an organic solvent, improves sample recovery. An alternative approach is to add enough water to cover the adsorbent material, and to extract the aqueous suspension several times with an immiscible organic solvent. Before solvent evaporation, colloidal silica is removed by filtration through a membrane filter. Common contaminants, such as phthalate esters and poly(estere)s, can be removed by chromatography on Sephadex LH-20 (section 11.3.3).

Rotation planar chromatography (centrifugal TLC) and forced-flow development provide higher resolution and shorter separation times for preparative-scale thin-layer chromatography (section 6.6.5), Table 11.1 [4,6,12,13,16]. These methods allow on-line detection for automated fraction collection as sample bands leave the layer. Fine-particle layers can be used in forced flow separations, to further enhance resolution with some loss in sample capacity. Although for complex mixtures, preparative-scale high-pressure liquid chromatography is generally preferred.

11.3 COLUMN LIQUID CHROMATOGRAPHY

Column liquid chromatography offers a wide range of options for preparative-scale separations that differ in resolving power, sample capacity, equipment requirements, and operating costs, Table 11.2 [1,4-6]. A distinction is possible in terms of the typical operating pressure. Classic or open column systems rely on capillary forces for transport of the mobile phase through the column. Since these forces are weak, the particle size must be large, and low efficiency, modest column lengths, limits the separation power. In addition, the separation time is compromised by the low flow rates achieved. Among the virtues of these columns are low costs, minimal equipment requirements, and high sample capacity. Stationary phase reuse is usually unnecessary, since it has a small influence on the cost of purification. For those cases that require

Table 11.2

Typical operational parameters for preparative-scale column liquid chromatography

Type	Particle size (μm)	Inlet pressure (atm)	Flow rate (ml/min)	Sample loading (g)
Open column	63-200	1	1-5	0.01-100
Flash chromatography	40-63	1-2	2-10	0.01-100
Low pressure	40-63	1-5	1-4	1-5
Medium pressure	15-40	5-20	3-16	0.05-100
High pressure	5-30	> 20	2-20	0.01-1

increased resolution, shorter separation times, or increased automation, the particle size must be reduced, and the column inlet pressure simultaneously increased, to maintain the optimum range of mobile phase velocities. These improvements can be obtained incrementally, as indicated in Table 11.2. Although a distinction is made between flash chromatography, low pressure, medium pressure, and high pressure operation, these are no more than regions of an operating continuum. The conditions indicated in Table 11.2 are considered typical and the boundaries flexible. One distinction that affects method selection is operating cost and equipment requirements. These increase in descending order in Table 11.2, and restrict availability in different laboratories.

11.3.1 Flash Chromatography

Flash chromatography is widely used for laboratory-scale fractionation of mixtures from organic synthesis, or for analysis, when only a modest increase in resolution over open column systems is required [4-6,17-19]. These techniques employ short columns packed with particles of an intermediate size (40 – 63 μm) combined with accelerated solvent flow achieved through modest pressure or suction. Compared with open column chromatography: separations are obtained in less time; isolated compounds are often purer, because resolution between bands is increased; and compounds that are degraded or altered during chromatography, are recovered in higher purity, because of the shorter contact time with the separation system. The main applications of flash chromatography are purification of synthetic products, isolation of target compounds from natural products, the simplification of mixtures prior to high resolution preparative (usually) liquid chromatography, and the fractionation of complex mixtures into simpler groups for analysis. Its primary virtue is low cost, since virtually no special equipment is required, and the stationary and mobile phases are inexpensive enough to be discarded after a single use, or can be recycled. Resolution is less than that obtained by medium and high-pressure liquid chromatography, but the operational costs and equipment needs are greater for these techniques. Flash chromatography is often employed as a pre-separation technique to remove particle matter and sample components that are either weakly or strongly retained on the separation column in medium and high-pressure liquid chromatography. This allows higher sample loads to be separated under more selective separation conditions, and avoids column contamination and

regeneration problems. The production costs of the isolated products are thus rendered more favorable.

Columns for flash chromatography are prepared as follows. A glass column of suitable length containing a small glass wool plug and a layer of acid washed sand, or with a glass frit at its base, is partially filled with sorbent using the dry packing or slurry packing technique. Incremental addition of the sorbent followed by tapping of the column with a hard object generally gives better results for dry packing than bulk filling of the column. After packing, the column is freed from trapped air and further consolidated by forcing several column volumes of a weak solvent through the column bed, until no further air bubbles are seen exiting the column and the bed is stable. It is difficult to pack wide diameter columns (> 5 cm) by dry-packing procedures, and in this case slurry packing is nearly always used. The column is partially filled with a small volume of weak solvent. A dilute suspension of the sorbent in the same solvent is added slowly, in increments, with excess solvent intermittently drained away. Periodic pressurization of the sorbent bed is used to aid consolidation. Tapping the side of the column with a hard object during packing is not generally recommended. The sample is added to the column in a small volume of solvent or adsorbed to a small amount of packing material. Finally, a thin layer of glass beads, acid-washed sand, or other inert material, is added to the top of the column to prevent disturbance of the column bed by solvent added for elution. The amount of free space above the packed bed must be sufficient to hold a volume of solvent equivalent to the fraction size collected, or a solvent reservoir must be inserted between the column and the pressure regulation valve [20]. The flow rate is adjusted to about 5 cm/min by application of pressure from a compressed gas cylinder or air pump, and controlled by the regulation valve. Pressures employed are typically less than 1 to 2 atmospheres with the various parts of the apparatus, Figure 11.1, held in place by springs, clamps, or screw-thread connectors. It is a wise precaution to use plastic coated glass columns, or a safety shield, to minimize the possibility of accidents. The column should not be allowed to run dry during the elution sequence. Radial compression columns have been used with large-scale flash chromatography systems [21]. Because of the limited operating pressure, columns are rarely longer than 30 cm, and 10 to 15 cm is recommended, unless longer columns are required to provide additional resolution. At a mobile phase velocity of about 5 cm/min, well-packed columns are expected to provide about 5 – 20 theoretical plates per cm of bed height, depending on the column packing density, and the quality of the sorbent material.

Silica, and to a lesser extent alumina, are the most common stationary phases used for the separation of low molecular mass organic compounds. Chemically bonded silica sorbents are used for the separation of polar organic compounds in the normal-phase and reversed-phase modes. Wide-pore, chemically bonded sorbents, are used for the separation of biopolymers [18,22]. Some separations require specially prepared stationary phases, such as silica gel impregnated with silver nitrate for the isolation of unsaturated compounds capable of forming charge transfer complexes with silver [23] (section 10.6.1), or silica and chemically bonded phases coated with cellulose tris(3,5-

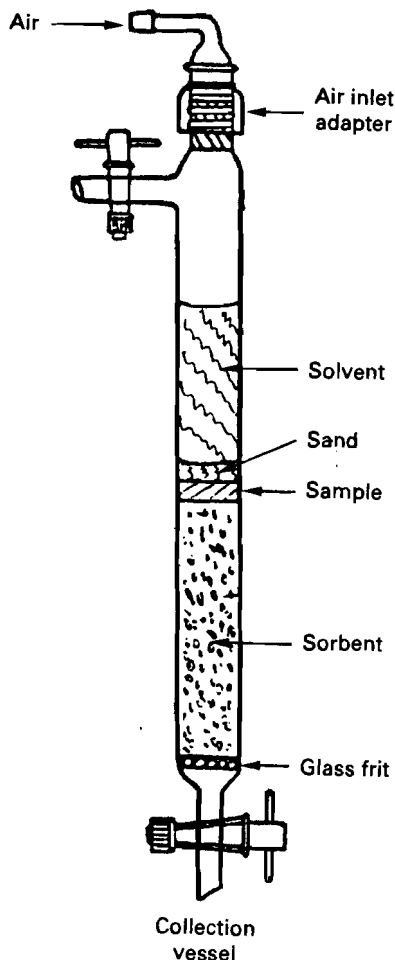


Figure 11.1. Apparatus for flash chromatography.

dimethylphenyl carbamate) to isolate single enantiomers from racemic mixtures [24] (section 10.4.2).

The sample is usually added to the column in a small volume of a weak solvent, and the solution forced into the sorbent bed forming a narrow sample band. For samples of low solubility in weak solvents, the sample is taken up in a strong solvent and added to a small amount of column packing or other inert support. The solvent is then stripped from the slurry under vacuum to produce a dry free-flowing powder (1-2 g sample / g sorbent) that can be added to the top of the column. It is important that the sample is completely dry (high vacuum used to remove last traces of solvent), and free of lumps, to obtain symmetrical separated bands. If the sample layer is relatively long compared

Table 11.3

Approximate sample-loading conditions for flash chromatography
(density of silica ≈ 0.45 g/ml)

Column diameter (cm)	Amount of silica gel (g)	Sample loading for a particular TLC resolution (g)		Typical fraction volume (ml)
		$\Delta R_F \geq 0.2$	$\Delta R_F \geq 0.1$	
<i>(a) Isocratic elution (bed height = 15 cm)</i>				
1	5	0.1	0.04	5
2	20	0.4	0.16	10
3	45	0.9	0.36	20
4	80	1.6	0.6	30
5	130	2.5	1.0	50
<i>(b) Stepwise gradient elution (bed height = 10 cm)</i>				
Column Diameter (cm)	Amount of silica gel (g)	Sample loading (g)	Typical fraction volume (ml)	
3	30	1 – 3	50 – 100	
4	55	3 – 8	100 – 200	
6	125	8 – 35	200 – 300	
8	250	35 – 60	200 – 300	
10	350	60 – 80	300 – 500	
14	700	80 – 150	300 – 500	

to the column bed length, then a stepwise solvent gradient must be used for elution to minimize band broadening.

There are no simple relationships between the sample amount that can be separated, the dimensions of the sorbent bed, and the volume and number of collected fractions. The sample capacity depends on the ease of separation of neighboring zones, the sorption capacity of the sorbent, and the method of elution. Wider columns and sorbents of high specific surface area are used to increase the sample capacity, Table 11.3. For stepwise gradient elution, it was assumed that the sample is separated into fractions of different polarity to estimate sample loads. Even for difficult samples, it is often more productive to use column overload conditions, combining fractions containing pure materials, and recycle those containing mixtures. Flash chromatography may lack the resolving power needed to separate the components of interest. In this case, a higher resolution technique, such as medium or high-pressure liquid chromatography is a better choice, perhaps using flash chromatography to isolate fractions containing the components of interest from other sample components.

Thin-layer chromatography is widely used to optimize separation conditions for silica gel flash chromatography. For isocratic separations, a mobile phase that provides a $R_F \approx 0.35$ for the zone of interest is chosen. If several zones are to be separated, then the solvent strength is adjusted such that the center zone has a $R_F \approx 0.35$. If all zones of interest are well separated from each other and from impurities ($\Delta R_F \geq 0.2$), then the solvent strength is adjusted so that the most retained zone of interest has a $R_F \approx 0.35$. For fractionation and large sample loads, it is critical that the most selective solvent composition for the separation is used. This can be quickly

identified from the Prisma model, a guided trial and error procedure using thin-layer chromatography (section 6.7.1). The same process can be used to identify solvent compositions suitable for recovery of sample zones in order of increasing polarity by stepwise gradient elution. For samples of wide polarity, a useful gradient is to start from a weak base solvent, such as hexane, and add to this various volume increments of a strong dipolar solvent (such as ethyl acetate, dichloromethane, chloroform, or acetone), terminating with the strong dipolar solvent. Then continue adding volume increments of a strong hydrogen-bond solvent (methanol, ethanol, 2-propanol) to the dipolar solvent, terminating with the strong hydrogen-bond solvent. Screening of the separation by thin-layer chromatography allows the solvent gradient to be trimmed and optimized to the requirements of individual separations. Predicting the number of fractions required at each step remains quite arbitrary, and is best conducted by monitoring the composition of each fraction as it is collected. When incrementing the composition of strong solvent in a binary mobile phase for silica gel, it is important to note that the solvent strength for the mixture has a steep curved profile (section 4.3.6.2). For compositions containing low volume fractions of strong solvent, the volume fraction of strong solvent should be incremented by small changes resulting in relatively large changes in retention, for example, 1 %, 3 %, 5 %, 10 % (v/v). At higher volume fractions of strong solvent, changes should be larger to produce a significant change in retention, for example, 30 %, 40 %, 60 %, 80 %, 100 % (v/v).

Water-soluble compounds, including biopolymers and easily ionized compounds, are generally isolated by reversed-phase chromatography. Optimization of solvent composition by thin-layer chromatography is possible, but predictions may be unreliable owing to differences in the sorption properties of the sorbents used to prepare the layers and those used for flash chromatography. A better solution is to pack a short (10 cm) metal column with the sorbent for flash chromatography and use high-pressure liquid chromatography to optimize separation conditions. Ideally, for isocratic elution a solvent composition should be chosen that provides a retention factor of 2 – 3 for the component of interest, or the most difficult to separate components of a mixture. For mixtures of wide polarity, stepwise solvent gradients are easily constructed and optimized by the same approach.

Separations by flash chromatography can be monitored on-line using UV-visible, refractive index, or evaporative light-scattering detection. Off-line monitoring, however, by collecting fractions that are subsequently combined based on the similarity of their composition is more common [4,21,25]. Thin-layer, gas or liquid chromatography, electrophoresis, bioassays, immunoassays, and spectroscopy (e.g. infrared and nuclear magnetic resonance) are suitable techniques to identify fractions that can be combined. Most of all, thin-layer chromatography is used because it is quick, portable, inexpensive and generally adequate for the task.

11.3.2 Dry-Column and Vacuum Chromatography

Dry-column chromatography is a variant of preparative-scale thin-layer chromatography with similar resolution but a higher sample capacity. A glass column or Nylon tube

is packed with a thin-layer sorbent, usually silica gel, to a height of 10 to 15 cm. Sample is added as a concentrated solution or preadsorbed onto a small amount of sorbent. Separation is achieved by developing with a suitable volume of solvent to reach the lower end of the bed. Suction at the bottom of the column and/or slight overpressure at the top may be required to supplement capillary forces in moving the mobile phase down the column. Separated bands are removed by extrusion, slicing (if a Nylon column is used), or by digging out, and the products freed from the sorbent by solvent extraction. The separation is fast, requires little solvent, and provides higher resolution than classical column techniques, owing to the use of sorbents with a smaller average particle size. It is suitable for the recovery of small quantities of material, since the loading capacity is only about 0.2 to 1.0 % w/w of the sorbent used depending on the difficulty of separating the bands of interest. Thin-layer chromatography provides a suitable technique for method development in most cases, although significant differences in separations can arise for mixed solvents, particularly when the solvent components differ in polarity and/or volatility. These differences result from the absence of a vapor phase in the dry-column technique. Nylon columns can be more difficult to pack than glass columns, particularly when longer lengths are used, but Nylon columns are easier to section, and allow colorless bands to be observed with a UV lamp. Glass columns, built up of segments connected by ground glass joints, simplify the extrusion process.

Dry-column chromatography is not a widely used today. Preparative-scale thin-layer chromatography or flash chromatography is generally preferred. Although separations are fast, the recovery of separated zones is slow and labor intensive compared with elution methods.

Vacuum chromatography can be taken to mean the operation of a short column under suction to accelerate solvent migration. Either a short column, or a Buchner filter funnel fitted with a glass frit, is dry-packed with sorbent [26]. The sorbent bed is consolidated by tapping the side of the column during filling, and pressing the top layer of the sorbent bed with a flat object, such as a stopper, while suction is applied at the other end. Consolidation is completed by releasing the vacuum and pouring a solvent of low polarity over the surface of the bed followed by restitution of the vacuum. If the column is packed correctly, the solvent front will descend the column in a horizontal line, otherwise the column should be sucked dry, repacked, and tested again. When all the solvent has passed through the column, residual solvent trapped between particles is removed by suction. A solution of the sample in a suitable (weak) solvent or preadsorbed onto a small amount of sorbent or inert material, such as Celite, is applied to the top of the column. The sample solvent, if used, is sucked gently into the column packing. A piece of filter paper with the same diameter as the inside diameter of the column, or funnel, is placed on top of the packed bed to prevent disruption of the bed during solvent addition. The column is eluted with appropriate solvent mixtures of gradually increasing solvent strength. Between solvent applications, the column is sucked dry, and the eluent collected in test tubes or round bottom flasks. A multiport manifold allows sequential fraction collection without having to disassemble the apparatus after each fraction is collected.

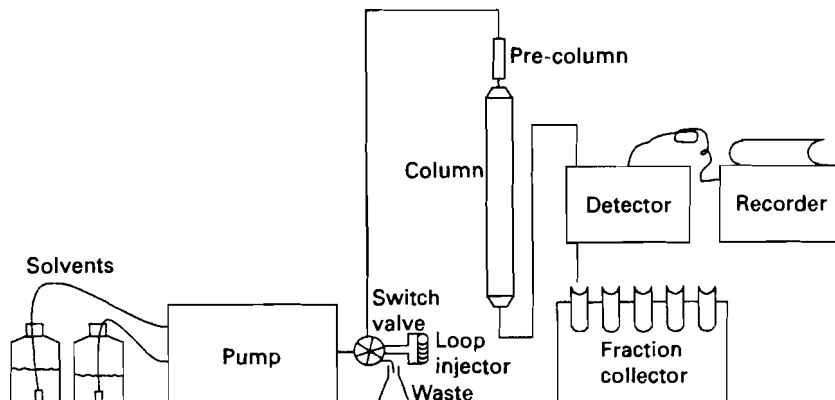


Figure 11.2. Apparatus for low- and medium-pressure preparative-scale liquid chromatography. (From ref. [6]; ©Academic Press).

Vacuum chromatography is simple, rapid, and convenient. Optimum sample loads are similar to flash chromatography. However, it is not unusual to use sample overload conditions to separate simple mixtures by stepwise gradient elution, or to simplify mixtures for further separation. Under these conditions the sample loads may reach 10 % (w/w), or even higher, of the bed mass.

11.3.3 Low- and Medium-Pressure Chromatography

Low-pressure liquid chromatography uses similar particle-size sorbents to flash chromatography, and its operation is only a little more complex. Column inlet pressures are similar, but a pump is used to maintain a constant mobile phase velocity, and sample introduction is usually by injection. On-line detection is frequently used together with an automated fraction collector. Medium-pressure liquid chromatography uses a similar experimental arrangement, Figure 11.2. It affords higher resolution and shorter separation times by using sorbents of a smaller average particle size, and higher mobile phase velocities, by operating at higher inlet pressures (see Table 11.1). These techniques are suitable for use with soft gels employed in the purification of biopolymers (section 11.3.7). As well as some natural and synthetic polymer gels, that provide different selectivity to conventional inorganic oxide-based sorbents, for the purification of low molecular mass organic compounds [3-6,27].

Columns for low- and medium-pressure liquid chromatography are made from strengthened glass with a plastic protective coating. The wall thickness and column internal diameter determine the maximum operating pressure. Narrow-bore columns (e.g. 1 cm) can be used at pressures up to about 50 atm., while wide-bore columns (> 10 cm) are restricted to pressures of a few atm. Columns are available in lengths from about 10 cm to 1 m (20-25, 30-35 and 44-50 cm are the most popular) with internal diameters from 1 to 10 cm (1-1.5, 2.5-4, 3.5-6.5 cm are the most popular). The wider

bore columns contain several kilograms of sorbent, and can separate sample loads of 15-100 g, depending on the ease of separation under concentration overload conditions. For narrow-bore columns, sample loads < 3 g are common, corresponding to a sample-to-sorbent mass ratio of about 1:25 for difficult separations, and lower ratios for easy separations. Columns can be coupled in series to create greater lengths, when needed, to improve resolution.

For low-pressure separations, inorganic oxides and chemically bonded sorbents of 40-63 μm , and for soft gels 25-100 μm , are typically used. For medium-pressure separations inorganic oxides, chemically bonded phases, and rigid or semi-rigid porous polymer sorbents with an average particle size of 15-25 or 25-40 μm are typically used. Larger particle sizes are appropriate for long or series-coupled columns, operated at modest pressures. Packed columns can be purchased, or prepared in the laboratory by dry or slurry packing, without the need for special equipment [4,6,27-30]. Dry packing is generally used for inorganic oxide sorbents. The tap-and-fill method (section 4.5.2.1) is suitable for inorganic oxides with an average particle size > 20 μm . Packing under nitrogen pressure is required for 15- μm particles. The column with a frit at its base is clamped vertically, and a reservoir attached at the top. The sorbent is introduced into the column by means of a funnel until the column is completely filled and the reservoir contains excess sorbent equivalent to about 10 % of the column volume, Figure 11.3. The column is not vibrated or tapped during the filling process. The system is connected to a nitrogen cylinder and 10-atm pressure applied (with the column outlet open) until the packing height reaches a constant value. The cylinder valve is closed, and the pressure allowed to bleed away. The reservoir is then removed, and the filled column conditioned by passing mobile phase through it until no more bubbles appear in the eluent, and the pressure stabilizes. Slurry packing techniques are used to pack chemically bonded phases (silica gel can be packed in this way as well). The slurry is prepared by suspending the sorbent in a solvent that is a good wetting agent. The slurry is slowly poured into the column to completely fill the column and partially fill a short precolumn attached to the separation column. The sorbent is allowed to consolidate for about 1 h and then connected to the eluent pump at the precolumn end. Solvent is pumped through the column, increasing the flow rate and pressure in steps, until the column operating pressure is reached, and the mobile phase flow rate stabilizes. The precolumn is then removed, and the column inlet fitting attached to the column. The column is then conditioned by passing mobile phase through the column to replace the packing solvent. Soft gels are first swollen in the mobile phase, and the slurry formed slowly poured into the column with stirring or tapping. Excess solvent is drained away, and the process repeated until the column is completely filled. The column is connected to the pump, and the bed consolidated by increasing the flow rate and pressure in increments, until the operating conditions are reached. Voids, which may appear at the inlet end of the column, are filled by adjusting the inlet fitting to the level of the column bed. Alternatively, further amounts of packing (or glass beads) are added to the column to restore the bed height to the desired position.

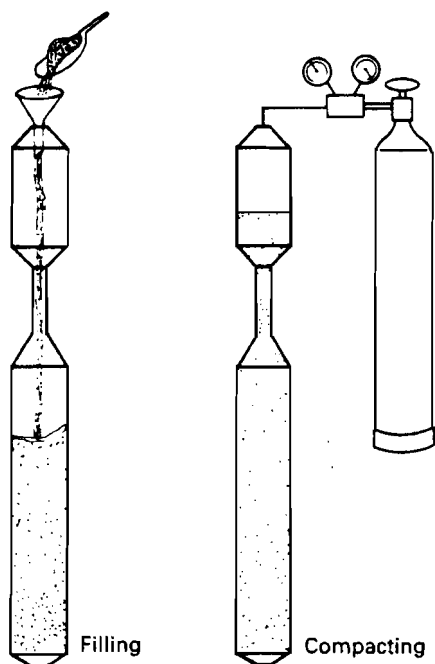


Figure 11.3. General procedure for dry packing medium-pressure, preparative-scale, liquid chromatography columns. (From ref. [6]; ©Academic Press).

The operating pressure (< 50 atm) and flow rates (5-180 ml/min) for the full range of columns used in medium-pressure liquid chromatography can be generated by low cost reciprocating-piston pumps with exchangeable pump heads. All connections between the pump, injector, column, and detector are made with Teflon tubes, or similar materials, having specially-designed plastic end fitting to simplify assembly of the equipment. The sample is usually injected on-stream using a valve injector or, for large sample volumes, through the pump. Loop injectors are suitable for injecting sample volumes up to about 100 ml. For low-pressure columns, the sample can be added to the head of the column by syringe or pipette, and the solution allowed to drain to the top of the column bed. For wide-bore columns, the design of the column inlet and end fittings is crucial to ensure an even distribution of the sample across the column diameter. This can be achieved with a fritted disc and plunger assembly (adjusting the plunger position allows column voids to be filled), Figure 11.4. A fraction collector is normally used instead of or in conjunction with a flow-through detector (a short path length UV detector or refractive index detector) for sample collection. Fractions are collected in test tubes (240 x 20 ml) or larger vessels (120 x 50 or 48 x 250 ml) on a time, volume, or peak threshold basis. In the absence of on-line monitoring, thin-layer chromatography can be used to group similar fractions.

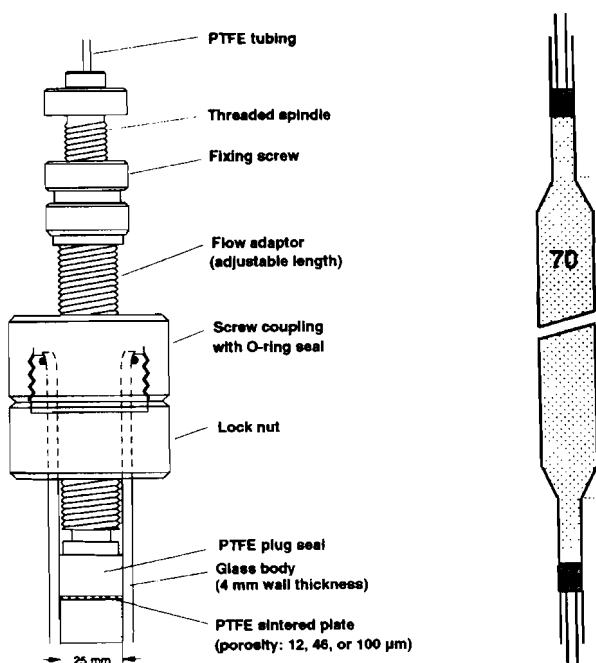


Figure 11.4. Expanded view of a column end fitting and conical shaped glass column for low- and medium-pressure, preparative-scale, liquid chromatography.

Separations should be performed with the most selective mobile phase. Suitable mobile phases are usually identified by thin-layer chromatography or analytical high-pressure liquid chromatography [31]. The surface area of thin-layer sorbents is higher than typical column sorbents ($\approx 2 \times$). Mobile phases of suitable strength for preparative-scale column separations, therefore, will have an $R_F \leq 0.3$ by thin-layer chromatography. Thin-layer chromatography can be used to select gradient elution conditions according to the location of zones in a standardized separation system [32]. Transfer of conditions from analytical high-pressure liquid chromatography to preparative-scale column chromatography is usually straightforward unless the difference in properties of the two column sorbents are significant. In general, normal-phase separation systems are preferred, since volatile organic solvents are easier to evaporate than aqueous solutions to recover the isolated compounds. Adequate solvent purity is an important consideration, since low level non-volatile impurities become concentrated during the evaporation step, and will contaminate the isolated compounds.

11.3.4 High-Pressure Chromatography

Higher operating pressures allow preparative-scale separations with improved resolution in a shorter time using smaller diameter particles, set against higher operating costs

Table 11.4

Some common terms used in preparative-scale chromatography

Parameter	Description
Cut points	Times, mobile phase volumes, or threshold values of the concentration when fraction collection begins or ends.
Cycle time	Time separating two consecutive injections.
Loading factor	Ratio of the sample size to the column saturation capacity.
Overload	Operation of the column under nonlinear isocratic conditions resulting from the separation of large sample sizes.
Phase ratio	The ratio of the fraction of the column volume occupied by the stationary and mobile phases ($F = [1 - \epsilon] / \epsilon$, where ϵ is the total porosity of the column). This definition is different from that used in analytical chromatography (section 1.4).
Production rate	Amount of product with a specified purity obtained per unit time.
Recovery yield	Ratio of the amount of the desired compound collected in the product fraction to the amount of sample injected on the column.
Recycle	A chromatographic process in which the fraction of the mixed zone is re-injected on-line, without intermediate collection and pooling.
Saturation capacity	Amount of component required to saturate the stationary phase. In adsorption, amount of component required to form a monolayer on the surface of a unit amount of adsorbent. In ion exchange, amount required to exchange all the ion sites on the stationary phase.
Specific production	Volume of mobile phase consumed per unit amount of product produced.
Throughput	Amount of sample introduced into the column per unit time.

and the need for more complex instrumentation [1-6,11,33-36]. Instruments for analytical separations can be adapted for use with semipreparative columns with internal diameters < 2.5 cm containing about 25-65 g of silica-based sorbent. These columns are operated at flow rates between 15 and 30 ml/min and are suitable for the purification of 50-100 mg of sample per injection. Special purpose instruments are required for operation at the higher flow rates required by larger diameter columns. These are usually only found in laboratories that specialize in preparative-scale separations, or have a re-occurring need for purification of difficult samples. A 30-cm column with an internal diameter of 10 cm, contains about a kilogram of sorbent, and can separate 10-100g of sample per injection. The actual sample load is strongly dependent on the ease of separation, and can be an order of magnitude higher for simple separations ($\alpha > 5$), or lower for difficult separations ($\alpha < 1.3$), when using sample overload conditions. Optimum flow rates are between 250 and 600 ml/min.

Some common terms used in preparative-scale liquid chromatography are summarized in Table 11.4. The production rate, specific production, or the recovery yield provide suitable objective functions to judge the relative success of individual methods. For efficient use of the separation system, the production rate and the recovery yield should be maximized. Invariably, this results in operating the column in an overloaded condition. Unfortunately, column operation under nonlinear conditions is complex, and optimum conditions are not as easy to predict as the less demanding, although less powerful, scale-up approach. To scale up an analytical separation, the same column packing, column length, and mobile phase velocity are used, and the column diameter increased

to achieve the desired sample capacity. The analytical separation should be optimized to maximize the separation factor between critical peaks to allow higher column loadings before the bands overlap.

11.3.4.1 Instrumentation

Wide bore columns operated at high production rates place different demands on the specification of instrument performance compared with analytical separations on narrow bore columns. For some applications, analytical instruments are easily adapted to preparative-scale operation, but in general, for all but occasional use and relatively small sample sizes, different equipment is required. Pumps for mobile phase delivery are required to generate isocratic or gradient mobile phase compositions at high flow rates and modest pressures, compared with analytical separations. Analytical pumps with a maximum flow rate of 10 ml/min are suitable for use with semipreparative columns, although the separation time may be compromised by the less than desirable flow rates. Some analytical pumps have exchangeable pump heads that extend the flow rate range to higher values, and are a better choice for dual analytical/preparative applications. For laboratory-scale preparative chromatography with a wide range of column types, a pump with a flow rate limit of at least 100 ml/min and a pressure limit of 200-250 atm is a reasonable compromise. Process-scale columns (I.D. > 10 cm) require flow rates of at least 2000 ml/min at operating pressures < 150 atm.

Conventional rotary injection valves with external loops are suitable for the relatively small sample sizes typically used with semipreparative columns. For sample volumes of several milliliters, it is easier to pump the sample onto the column using the eluent pump, or preferably, a dedicated injection pump. Wide bore columns require a header device to distribute the sample and mobile phase over the cross-section of the column at the inlet, and as a collector at the exit [30,37-40]. The header consists of a frit and a distributor, and is usually part of the column inlet and exit fittings. The frit serves to prevent movement of the packed bed out of the column, and conceivably plays some role in the flow distribution. The distributor is typically a flat porous disc or a non-penetrable disc with branching channels, usually with a void space above it, to promote rapid radial flow. The distributor is designed to ensure that all streamlines from the point of injection to the column packing (or from the collection region to the column exit) have identical transit times and a flat radial velocity flow profile. Path length and flow velocity heterogeneity at the head or bottom of wide bore columns can be a significant source of band broadening. Significant viscosity differences between the sample solution and the mobile phase can affect the shape of separated bands through viscous fingering. This results from the penetration (fingering) of the less viscous solution into the more viscous solution, as the two regions penetrate the packed bed.

A wide linear response range and high flow rate capability, are desirable features in a detector for preparative-scale chromatography. Problems with temperature stability (refractive index) and pressure pulses (UV detector) are less important because of the high sample concentrations present in the mobile phase. Some analytical detectors have interchangeable flow cells, which allow replacement of the analytical cell with one of

shorter path length to reduce sensitivity. Preparative flow cells usually have wider bore inlet and outlet connections to accommodate high mobile phase flow rates. For the same reason, wide bore capillary tubing (0.4-0.6 mm I. D.) is used for column connections, instead of the narrow bore tubing used with analytical columns. It is often convenient to operate analytical detectors with a low-dead volume flow splitter, so that only a few percent of the total column eluent passes through the detector. This is essential if a mass spectrometer is used for mass-selective detection of target compounds for automated fraction collection [11,41-44]. A mass spectrometer is an expensive detector for preparative-scale chromatography, but has a number of useful features in fully automated systems, where UV detection may lack the specificity to identify selected products for collection. With mass-directed fraction collection, no post-purification screening and pooling is required to identify product fractions, and tighter control is maintained over fraction size and number of collected fractions. The latter is critical for unattended operation, if excessively large fraction beds are to be avoided.

11.3.4.2 Columns

Wide bore columns must be heavily walled compared with analytical columns if they are to be used with small diameter particles, or alternatively, they must be operated at significantly lower inlet pressures, reflecting their lower bursting pressure [2,45,46]. Some stainless steel columns with an internal diameter of 5 cm, for example, have a maximum operating pressure of about 100 atm. Compression fittings may not seal adequately to wide diameter columns at high pressures, and flanged end fittings are generally used. They are either bolted or fastened with special collars, which can withstand high pressures.

Dry or slurry packing of large diameter columns is more difficult than those of conventional diameters owing to the scale of the packing process, and the high flow rates and pressures required to consolidate the packed bed [36,45-48]. Anecdotal evidence indicates that columns of large internal diameter are less stable than analytical columns. This is attributed to the packing density being too low at the beginning of column operation. These problems are associated with the application of insufficiently intense vibration of the bed during dry packing, or an insufficiently high flow velocity, and hence packing pressure, during slurry packing. During dry packing, the coarse particles tend to segregate close to the wall region, resulting in a higher local-mobile phase velocity at the wall. The packing density for slurry packed columns is higher at the wall, while the core region is more permeable. Inefficient dissipation of frictional heat generated by the flow of mobile phase in wide bore columns can be a problem, since the stainless steel mantle and packed bed have different thermal expansion properties. Through repeated thermal expansion and contraction, the packed bed structure changes, and a void is produced at the top and along the walls of the column.

The preferred solution to these problems is to continuously adapt the column volume to the changing bed volume by radial or axial compression, Figure 11.5 [45-48]. Axial compression can be exerted from above with an adjustable column head, or from the bottom with a fixed or floating piston. Radial compression is achieved by applying

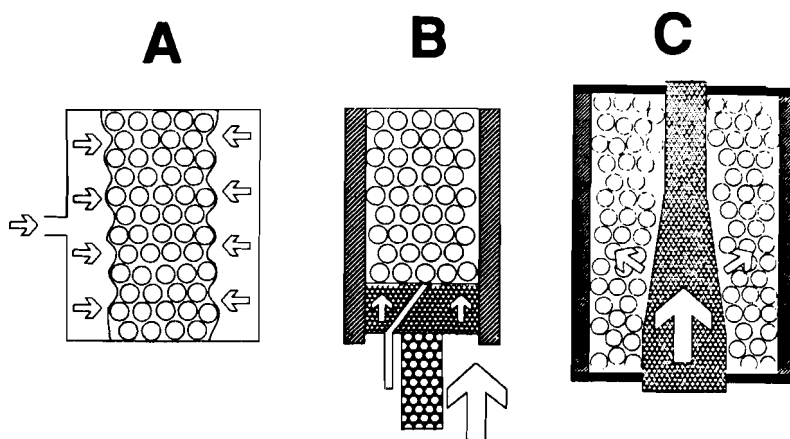


Figure 11.5. Schematic diagram of continuous compression columns for high-pressure Preparative-scale liquid chromatography. The arrows represent the force directions. A = radial compression, B = dynamic axial compression, and C = annular expansion.

hydraulic pressure between a flexible mantle and rigid container. A combination of axial and radial compression is possible by inserting a wedge or plunger in the center of the column top, which is screwed down, as the column conditions require. For columns with internal diameters < 2.5 cm, continuous bed chromatographic columns with a biporous structure (monolithic columns, section 4.2.7) represent a recent and promising development [49-51].

Dynamic axial compression columns consists of a cylinder, which is approximately three times longer than the column length desired, and a sliding piston with appropriate fittings to prevent leaks between the piston and the barrel [2,45,46,48,52]. Column bodies with internal diameters of 2.5, 5 and 7.5 cm are available for laboratory use, and in various diameters up to 80 cm, for process-scale applications. To prepare a column, the cylinder is first filled with a slurry of the packing material, the injection head bolted to the cylinder, and the piston slowly raised forcing the solvent out of the cylinder. A frit in the injection head fitting retains the packing during the compression phase. When the process of compression is complete, the cylinder is locked in position to prevent it from being pushed backwards, if the mobile phase inlet pressure exceeds the piston pressure used to consolidate the bed. An alternative design employs a floating piston, which uses the mobile-phase inlet pressure to continuously consolidate the column packing. After the separation, the injection head is unbolted, and the packing pushed out by the piston. Alternatively, a spring compression system that allows the column to be removed from the piston, and the system reused to pack another column, can be used [52]. The key parameters that affect the production of homogeneously packed and stable column are the packing material, the choice of slurry solvent, the slurry concentration, and the packing pressure [48,53-57]. There are no universal packing methods, and general approaches are adjusted to the requirements of individual packing materials.

Radial compression columns consist of a plastic cartridge closed at both ends by a frit and a stream distributor [58,59]. The cartridge is dry packed with packing material and inserted into a steel cylinder forming a seal at both ends. A hydraulic fluid is introduced under pressure between the plastic cartridge and the steel cylinder, producing compression of the bed. For optimum performance, the hydraulic pressure should be about 150 % of the inlet pressure required to obtain the desired mobile phase velocity for the separation. For coarse particles, the separation performance is similar to slurry packed analytical columns prepared from the same packing material. In current practice, dynamic axial compression columns are generally preferred to radial compression columns.

11.3.4.3 Scale-Up

The scale-up approach to preparative high-pressure liquid chromatography is quite straightforward, since the optimum separation conditions for the preparative-scale separation are extrapolated from a separation developed on an analytical column [1,33,34,60-63]. The analytical separation is optimized to maximize the separation factor between product peaks and product peaks and impurities. In addition, the retention factor for the product peaks is made as small as practical ($k < 3$) to conserve mobile phase, and to reduce the cycle time to facilitate multiple injections in the preparative-scale separation. Where possible, the mobile phase should contain components that are easily removed from the products to minimize the work-up of the collected fractions. In most cases, evaporation is used to separate products from the mobile phase. Consequently, the presence of low volatility additives and buffers are undesirable, and should be replaced by volatile components, where possible. Solubility of the sample in the mobile phase is an important consideration, since this may limit sample throughput. The higher solubility of organic compounds in organic solvents often results in normal-phase separations being preferred. It is notable that the preferred conditions for preparative-scale separations are not necessarily the same, as the conditions preferred for analysis.

Once separation conditions are established, the maximum sample load is determined. To minimize sample waste this is determined for the analytical column. The sample size (volume or mass) is increased in increments until the product bands are just touching, but still separated from impurity peaks [60,64-66]. This corresponds to the upper region of the linear portion of the sorption isotherms for the products, or to a slight overload condition. For difficult separations, partially overlapped peaks may have to be accepted to obtain acceptable sample throughput, in which case, product purity is maintained by collecting the outside portions of the product peaks and recycling the middle portion, Figure 11.6.

To scale-up the separation conditions to preparative chromatography, a preparative-scale column of the same length, but with a wider bore than the analytical column, and packed with the same (or similar) stationary phase material, is selected. The operating conditions for the preparative-scale column are estimated from the scaling factor, which is determined by the ratio of the volume of stationary phase in the two columns. This

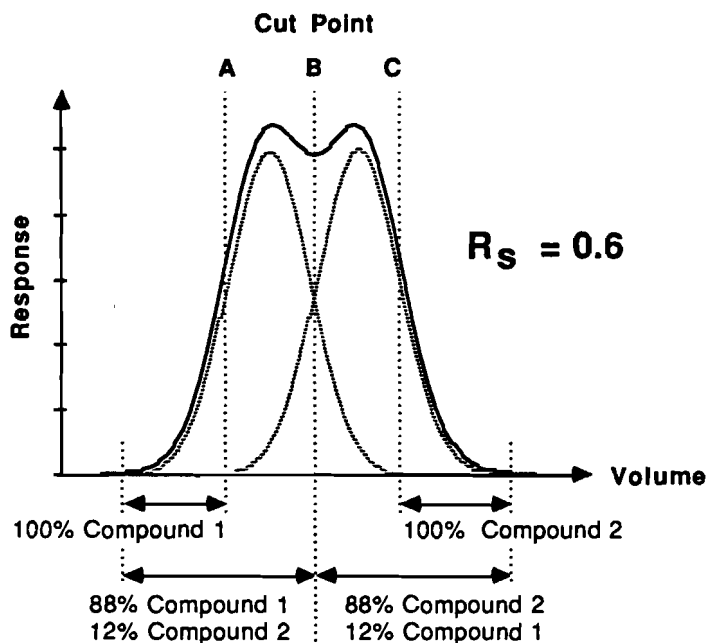


Figure 11.6. Peak shaving technique for collection of pure product when the separation selectivity is inadequate for acceptable sample throughput.

is equivalent to the ratio of the square of the column diameters. The sample load (both mass and volume) and mobile phase velocity required for equivalent separation conditions on the preparative-scale column are obtained by multiplying the analytical column conditions by the scaling factor, Figure 11.7. The irregular shaped peaks in the preparative-scale separation are due to detector saturation and not column overload. In those cases where the available instrumentation does not allow use of the calculated flow rate, the highest available flow rate is used. In this case, a reduced production rate owing to lower column efficiency, and longer separation times has to be accepted.

11.3.4.4 Column Overload Conditions

The scale-up approach to preparative chromatography is based on the theory for linear chromatography. Since there is a limit to the column size that can be prepared with the same efficiency as analytical columns, the only practical way to further increase sample throughput is to overload the column. A column is considered overloaded when retention factors obtained at low sample concentrations, change by more than 10% as the sample size is increased. A column might be overloaded either by increasing the sample concentration while maintaining a constant injection volume (concentration overload), or by increasing the injection volume while maintaining a constant sample concentration (volume overload) [64-66]. Under volume overload conditions, the sample concentra-

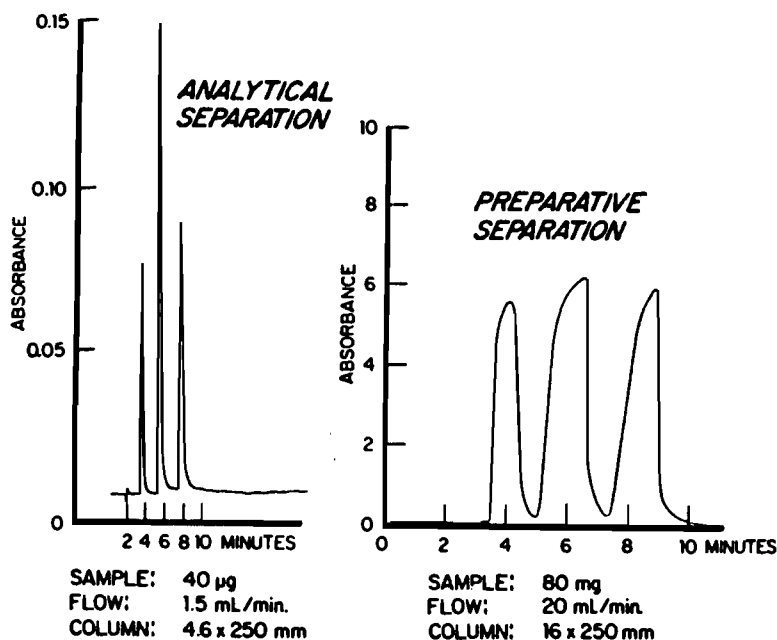


Figure 11.7. Application of the scale-up approach for the isolation of bilirubin isomers by high-pressure column liquid chromatography.

tion is kept constant and is confined to the linear range of the adsorption isotherm, but the volume injected is very large. For rectangular injection pulses of constant height (concentration) and increasing width (volume), the elution band becomes higher and wider. Ultimately, it becomes flat topped but remains symmetrical, Figure 11.8 [65]. For concentration overload, a small sample volume is injected, but its concentration exceeds the linear range of the adsorption isotherm. Accordingly, the band profile broadens and becomes assymetric; in the case of Langmuir type isotherms, the profile becomes close to a triangle, with an almost vertical front and a slanted tail.

The production rate in the elution mode increases with increasing volume and/or concentration of the sample injected on the column [66,67]. Since the bandwidth of all components increases in both cases, there is a limit to the sample size that can be used effectively. When the sample size increases from the very low levels typically used in analytical applications, the recovery yield remains constant, and the production rate increases linearly with increasing sample size. When the band of the compound of interest touches its neighbor, the recovery yield starts to decline with increasing sample size, since the wings of the elution band must be clipped to eliminate contamination. Eventually, a maximum value for the production rate is reached, but since the separation is carried out under nonlinear conditions, optimum separation conditions are no longer predictable from data obtained under analytical conditions.

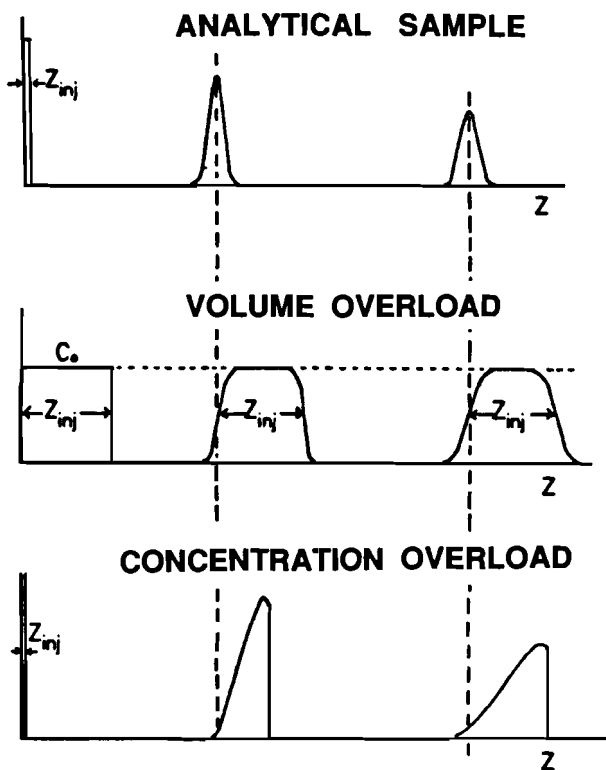


Figure 11.8. Development of peak profiles during migration along the column for analytical and overload samples. (From ref. [65]; ©Elsevier).

The properties of chromatographic bands under nonlinear conditions are difficult to predict and to model compared with linear chromatography [65-77]. At high sample concentrations equilibrium isotherms are nonlinear, the concentration in the stationary phase increasing either faster or more slowly than the concentration in the mobile phase. Accordingly, solutes at different concentrations tend to move along the column at different velocities, and either the front or rear of the band will become steeper. Peaks often have sharp fronts and long, diffuse rear boundaries. The position of the high concentration front recedes with increasing sample size, while the end of the peak tail holds the same position, identical to the peak position observed in linear chromatography.

Solute bands compete with each other for access to the adsorption sites on the stationary phase, resulting in interactions between bands and profile changes during their migration along the column. The amount of one component adsorbed at equilibrium depends on the concentration of all system components [66,78-81]. Compared with the results for a single component, displacement and tag-along effects

are observed. These effects control the shape of the profiles for individual bands of a mixture when these bands remain unresolved for at least part of their time on the column. The intensities of the displacement and tag-along effects depend essentially on the sample size, the sample composition, and the parameters of the competitive equilibrium isotherm for the components involved. The intensity of the tag-along effect is measured by the length of the concentration plateau of the second component left behind by the first component. This plateau results from the fact that the velocity associated with a certain concentration of the second component is a decreasing function of the local concentration of the first component. An increase in the ratio of the column-loading factor for the second component, relative to the first, will lead to an increase in the intensity of the tag-along effect. The opposite is true for the displacement effect, which affects the first eluting peak of the pair, and results in its elution as a characteristic concentration plateau.

To describe the peak shapes of a separation under overload conditions a clear understanding of how the competitive phase equilibria, the finite rate of mass transfer, and dispersion phenomena combine to affect band profiles is required [11,66,42,75,76]. The general solution to this problem requires a set of mass conservation equations; appropriate initial and boundary conditions that describe the exact process implemented; the multicomponent isotherms; and a suitable model for mass transfer kinetics. As an example, the most widely used mass conservation equation is the equilibrium-dispersive model

$$(\partial C / \partial t) + F(\partial q / \partial t) + u(\partial C / \partial z) = D_{ap}(\partial^2 C / \partial z^2) \quad (11.1)$$

where C is the solute concentration in the mobile phase, q the concentration in the stationary phase, t time, z distance along the column, F phase ratio (see Table 11.4), u the mobile phase velocity, and D_{ap} the apparent dispersion coefficient, which accounts for the finite column efficiency ($D_{ap} = uL / 2N$ where L is the column length and N the column plate count for linear conditions). The isotherm model provides the connection between the concentration of each component in the mobile and stationary phases (section 1.5.5), and the specific initial and boundary conditions close the system of partial differential equations. There is no algebraic solution to the system of partial differential equations obtained, and numerical solutions are necessary.

Prominent models for estimating peak profiles carry out a differentiation of the equilibrium isotherm with approximations for the mass transfer contribution. The equilibrium-dispersive model, above, assumes that all contributions due to non-equilibrium can be lumped into an apparent axial dispersion term. It further assumes that the apparent dispersion coefficient of the solutes remain constant, independent of the concentration of the sample components. For small particles, these approximations are reasonable for many applications. The ideal model assumes that the column efficiency is infinite. There is no axial diffusion, and the two phases are constantly at equilibrium. The band profiles obtained as solutions are in good agreement with experimental chromatograms for columns with $N > 1000$ having high loading factors. On the other

hand, the agreement with experimental results is poor for small values of N with low loading factors. In this case, the contributions from mass transfer kinetics and axial dispersion to the band profiles become significant compared with the influence of the nonlinear isotherm. The lumped kinetic models combine the mass balance equation with a kinetic equation, relating the concentration change of each component in the stationary phase to its concentration in both phases, and to the equilibrium concentration in the stationary phase. Results obtained are generally similar to the equilibrium-dispersive model, with some exceptions noted [64]. All the above models require extensive computational time, and include experimental data that is difficult and time consuming to acquire. In general, the agreement between the models and experimental band profiles is reasonable.

Due to the complex interactions among the large number of parameters that affect the operation of a column under overload conditions, it is difficult to establish a general guide to the selection of suitable operating conditions for different samples [11,46,64,65,80-82]. The following general observations are applicable to most cases. The column plate count should be as high as possible, and separations carried out using concentration overload conditions. The production rate of a purified fraction from the same sample increases with $(\alpha^2 - 1)$, where α is the relative retention of the desired compound and its neighboring impurity (i.e. the ratio of the origin slopes of their equilibrium isotherms). Preparative columns should be operated at higher mobile phase velocities than is typical for analytical columns. There is an optimum value of d_p^2 / L (d_p is the particle diameter and L the column length) for which the production rate is a maximum. This ratio is not easy to calculate for a given set of separation conditions, but is more important than specifying values of the individual parameters themselves.

11.3.5 Displacement Chromatography

Displacement chromatography offers an alternative to elution chromatography for preparative-scale separations under nonlinear conditions [10,66,82,83]. It has found limited success for the purification of biopolymers by reversed-phase [10,83,84] and ion-exchange chromatography [85-88], but is not widely used for the purification of small molecules [89-91]. It has the potential for greater use, but remains a minor technique compared with elution chromatography.

In displacement chromatography, the substances are resolved into consecutive zones (displacement train) of the respective pure substances, which leave the column in the order of their affinity for the stationary phase, rather than into peaks, like elution chromatography. Advantages of the displacement mode for purification are that the separated products are obtained in higher concentration than in the elution mode, solvent consumption is less, and band tailing is reduced as a result of the self-sharpening boundaries obtained. Separations are generally faster than for elution, but the cycle time is often greater, owing to the need to regenerate the column (rinse away the displacer and equilibrate with the initial mobile phase) between each injection.

In displacement chromatography the column is first equilibrated with mobile phase (referred to as the carrier), chosen for its low solvent strength in the separation system,

and as a good solvent for the sample. After equilibration, the sample is introduced into the column, usually dissolved in the mobile phase, where it is concentrated at the head of the column. Thus, it is important that the stationary phase exhibits strong retention of the sample components with the carrier solvent as eluent. In the next step, a concentrated solution of the displacer (usually in the carrier) is continuously pumped through the column. The displacer is a substance with a higher affinity for the stationary phase than any of the sample components, and causes the sample components to move down the column at speeds determined by the displacer front velocity. Stronger adsorbing sample components displace from the stationary phase surface those having weaker retention until the separation is achieved. The mixture separates into a displacement train, and once fully developed, the train is composed of adjacent rectangular bands of near uniform concentration, all moving at the same velocity. Following the breakthrough of the displacer front, the column needs to be regenerated and conditioned for further use.

Critical parameters for a separation are the choice of the displacer and its concentration, the loading factor, and the column efficiency. The choice of displacer is probably the most critical step, and is usually identified in an empirical screening procedure, often by evaluating compounds similar to the sample, or polyelectrolytes in the case of ion-exchange. For correct development of the displacement train, the adsorption isotherm of the displacer must lie above those of all the sample components. The concentration of the displacer controls the separation time and the concentration of the sample zones (the length [but not the height] of the separated zones is proportional to the sample amount). The displacer concentration is optimized for each system by a screening experiment. The choice of carrier solvent is less critical. It must be a good solvent for the displacer and the sample, as well as providing high retention factors for the sample in the separation system. To minimize the effects of axial dispersion, the optimum flow rate is usually low, compared elution chromatography. The column must be long enough for the development train to be fully formed, but need not be longer than this minimum length for a given sample size. Since the length of the zones in the displacement train increase with increasing sample amounts, longer columns are required to accommodate larger samples. Typically, the column length is fixed and the maximum sample size is established in a screening experiment. Injection of too large a sample for the column results in lower product purity owing to incomplete formation of the displacement train.

11.3.6 Simulated Moving Bed Chromatography

Simulated moving bed chromatography overcomes the limitations of batch processes (e.g. elution and displacement chromatography), namely, discontinuous operation, inefficient use of the stationary phase, and high product dilution. Simulated moving bed chromatography was developed as a process-scale operation, where its economic benefits have been clearly demonstrated. In recent years, it has been downscaled to a pilot-plant and laboratory-scale processes, and is receiving increasing attention in the fine chemicals and pharmaceutical industries for the purification of high value added products (e.g. enantiomers) and in biotechnology. Simulated moving

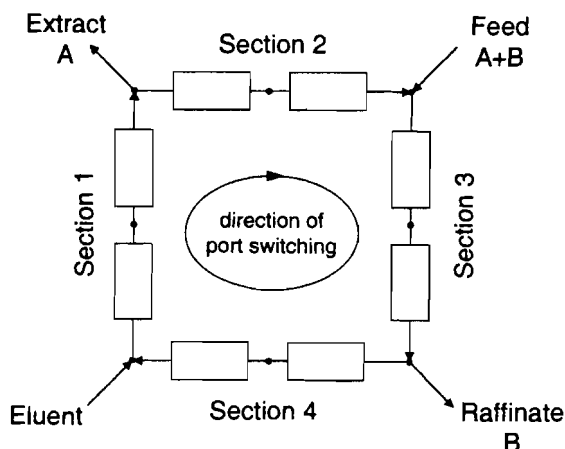


Figure 11.9. Schematic diagram of a simulated moving bed chromatography for the separation of a binary mixture ($A + B$), where B is the component with the lower affinity for the stationary phase. (From ref. [94]; ©Elsevier).

bed chromatography is a complex process, and laboratory-scale operations are often performed to assist in scale-up operations. It is unclear, however, whether the favorable production economies of simulated moving bed chromatography are sufficient to warrant the extensive effort required for optimization in laboratories that need to purify modest amounts of a range of different mixtures.

The simulated moving bed process is a way of implementing a true countercurrent chromatographic process in which the stationary and mobile phases are considered continuous phases moving in opposite directions. A true moving bed process is mechanically difficult to implement with liquid and solid phases. The simulated moving bed system consists of several identical columns (in most cases 6-12), which are connected in series, Figure 11.9 [92-95]. Each column is connected through a number of valves (depending on design) which can be switched to an open or closed position. A recycling pump inside the column circle delivers the mobile phase through all columns. Two additional pumps constantly inject the sample stream (feed) and fresh mobile phase, and two (or more) additional pumps withdraw the product streams (raffinate and extract flows). With multiple pumps, the internal recycle and all external flow streams are controlled and monitored. The countercurrent movement of the stationary and mobile phases is simulated by simultaneously switching the external and recycle flow streams, at discrete time intervals, and in the same direction. The periodic switching simulates movement of the solid phase by steps, and effectively the countercurrent movement of the stationary phase. In the "VARICOL" process, the flow streams are shifted asynchronously, changing the column allocation in the time domain between the external flow streams [96]. In both cases, the less retained sample component must move in the same direction as the mobile phase in all columns, and

the more retained component in the opposite direction. Although simulated moving bed technology provides a number of advantages leading to cleaner, smaller, and faster processes, the main disadvantage is the limitation to either the separation of binary mixtures, or recovery of one component from a multicomponent mixture. The purification of ternary mixtures is possible, but the systems are significantly more complex to design and operate [97,98]. In general, simulated moving bed processes are operated under isocratic conditions. Separation performance, however, can be further improved by gradient operation in order to optimize the retention behavior of the mixture components in each section of the unit [99,100].

In the simulated moving bed chromatograph, the columns are considered distributed among four sections based on the location of the external flow streams, Figure 11.9. Section 1 is located between the mobile phase inlet (eluent) and the product stream containing the product with a higher affinity for the stationary phase (extract). Its function is to desorb the more strongly retained product. Section 2 is located between the extract line and the sample inlet stream (feed). Its function is to desorb the product with a lower affinity for the stationary phase. Section 3 is located between the feed and the product stream containing the component with a lower affinity for the stationary phase (raffinate). Its function is to adsorb the product with a higher affinity for the stationary phase. Section 4 is located between the raffinate and eluent streams. Its function is to adsorb the product with the lower affinity for the stationary phase. In the case of a binary mixture, the sample stream carries the two components into section 3. The component with the lower affinity for the stationary phase is eluted from the column(s) in the raffinate before switching the external flow streams. The component with higher affinity for the stationary phase remains bound to the stationary phase. Because of flow switching, it remains in the column until the column reaches section 1, where it is eluted in the extract stream. With proper working conditions, the more retained product is not eluted from the column(s) of section 3, and the less retained component does not adsorb in the column(s) of section 2. In section 4, the least retained product is adsorbed on the column(s), so the mobile phase is cleaned, and can be recycled to section 1.

Totally empirical optimization of a simulated moving bed process is difficult and time consuming. Computer simulations are generally used for the design and optimization under linear [92,94,101-103] and nonlinear [95,102-105] operating conditions. Efficient operation requires control and knowledge of a large number of parameters. The most critical are: the column length and internal diameter; number of columns per section; stationary phase type and particle size; eluent composition; eluent, feed, raffinate, extract, and recycle flow rates; column pressure drop; and switching time intervals. For simulation of the separation process, a set of mass balance, kinetic, and isotherm equations are required for each component of the feed, and fluid dynamics equations for the columns. The model coefficients for these equations are obtained by exploratory experiments on a single column. Thus, it is important that the individual columns in the separation unit have (near) identical properties [104]. Simulated moving bed chromatography is a process operating design that is equally applicable to supercritical fluid [106,107] and gas [108-110] chromatography, as for liquid chromatography.

11.3.7 Sorbents for Biopolymer Separations

Sorbents used for preparative-scale chromatography can be placed into two categories. The larger group contains sorbents of similar morphology, surface chemistry, and mechanical strength to those employed for analytical separations (section 4.2). For preparative-scale applications, the particle size might be larger, but all other properties are virtually identical. For analytical applications the stationary phase is reused many times, but this is not always possible for preparative-scale applications where, in addition, the quantity of stationary phase employed for purification can be orders of magnitude higher. Economic considerations dictate that sorbents of a lower cost (and lower specification) are useful for the initial isolation of semi-pure products from crude samples. In addition, sorbents with a configuration that concurrently optimizes separation speed and sample capacity are desirable for preparative-scale chromatography, but are of less interest for analysis. Most sorbents in this second category evolved from the need for alternative approaches for the purification of biotechnology products and the isolation of biopolymers.

Sorbents for preparative-scale chromatography can be categorized according to composition, structure and mechanical strength. Those termed soft gels, are suitable for use at low pressures (< 3-5 atm) because of dimensional instability at higher pressures. Semi-rigid gels can be used at low to medium pressures (< 15-30 atm), and provide higher performance and shorter separation times. These gels require solvation by an appropriate solvent to create a defined pore structure, suitable chromatographic properties, and a favorable sample capacity.

11.3.7.1 Natural and Synthetic Soft and Semi-Rigid Gels

Starch, agarose, and cellulose are examples of natural polymers which spontaneously form pore networks when precipitated from a suitable solvent [6,111-116]. Dextran also forms a defined pore network in solution, but only after crosslinking [6,113-117]. Agarose and crosslinked dextran, and to a lesser extent cellulose, are the most important natural gels for preparative-scale chromatography based on size exclusion, Table 11.5. These sorbents have a high concentration of surface hydroxyl groups providing biocompatibility, as well as facilitating chemical modification of the sorbent surface. Common applications are the separation of biopolymers by size exclusion or ion-exchange chromatography (after the introduction of ionic functional groups), and as a support for the preparation of affinity sorbents (section 11.3.8).

Agarose is a linear copolymer of β -D-galactopyranose and 3,6-anhydro- α -L-galactopyranose with a low concentration of sulfate groups prepared from agar, isolated from marine algae. Non-crosslinked agarose beads (2-100 μ m) are produced by a simple emulsion-gelation procedure. Reaction with 2,3-dibromopropanol, epichlorohydrin or divinyl sulfone under strongly alkaline conditions forms crosslinks within the bundles of agarose chains, stabilizing the bead structure, while preserving the porosity. The pore size (5 – 100 nm) is controlled by the agarose concentration in the solution from which it is gelled. Agarose is stable in water and salt solutions at pH 4-9. Phenyl and octyl

Table 11.5

Natural soft and semi-rigid gels for the separation of biopolymers

(A / D agarose-dextran copolymer, D / bA dextran-methylene bisacrylamide copolymer)

Type	Polymer	Properties Particle size (μm)	Fractionation range
Sepharose 6B	Agarose	45-165	$1 \times 10^4 - 4 \times 10^6$
4B		60-140	$6 \times 10^4 - 2 \times 10^7$
2B		60-200	$7 \times 10^4 - 4 \times 10^7$
CL-6B	Crosslinked	45-165	$1 \times 10^4 - 4 \times 10^6$
CL-4B	Agarose	60-140	$6 \times 10^4 - 2 \times 10^7$
CL-2B		60-200	$7 \times 10^4 - 4 \times 10^7$
Superose 12	Crosslinked	34	$1 \times 10^3 - 2 \times 10^6$
6	Agarose	34	$5 \times 10^3 - 4 \times 10^7$
Sephedex G-25	Crosslinked	10 - 40 or 40 - 120	$1 \times 10^3 - 5 \times 10^3$
G-50	Dextran	10 - 40 or 40 - 120	$1.5 \times 10^3 - 3 \times 10^4$
G-75		10 - 40 or 40 - 120	$3 \times 10^3 - 7 \times 10^4$
G-100		10 - 40 or 40 - 120	$4 \times 10^3 - 1 \times 10^5$
G-150		10 - 40 or 40 - 120	$5 \times 10^3 - 3 \times 10^5$
G-200		10 - 40 or 40 - 120	$5 \times 10^3 - 6 \times 10^5$
Superdex 75	A / D	22 - 44	$3 \times 10^3 - 7 \times 10^4$
200	A / D	22 - 44	$1 \times 10^4 - 1 \times 10^5$
Sephacryl S-100 HR	D / bA	20 - 75 or 40 - 105	$1 \times 10^3 - 1 \times 10^5$
S-200 HR	D / bA	20 - 75 or 40 - 105	$5 \times 10^3 - 2.5 \times 10^5$
S-300 HR	D / bA	20 - 75 or 40 - 105	$1 \times 10^4 - 1.5 \times 10^6$
S-400 HR	D / bA	20 - 75 or 40 - 105	$2 \times 10^4 - 8 \times 10^6$
S-500 HR	D / bA	20 - 75 or 40 - 105	$2 \times 10^4 - 3 \times 10^9$
Cellufine GCL-90	Cellulose	45 - 84 or 45 - 105	$5 \times 10^2 - 4 \times 10^4$
GCL-300		45 - 84 or 45 - 105	$1 \times 10^3 - 1 \times 10^5$
GCL-700		45 - 84 or 45 - 105	$2 \times 10^4 - 3 \times 10^5$
GCL-1000		45 - 84 or 45 - 105	$2 \times 10^3 - 6 \times 10^5$
GCL-2000		45 - 84 or 45 - 105	$2 \times 10^3 - 3 \times 10^6$

derivatives of crosslinked agarose are used for separations by hydrophobic interaction chromatography (section 4.3.5).

Dextran, a branched homopolymer of D-glucose, is produced in bead form from a water-oil suspension to which a suitable crosslinking reagent (see agarose) is added. Point crosslinking of the dextran chains stabilizes the bead structure, forming soft gels with a pore size determined by the relative concentration of dextran and the crosslinking reagent. A low concentration of reactants produces materials with a high porosity at the expense of rigidity. Stronger materials are available by reaction of allyldextran with methylenebis(acrylamide) as the crosslinking reagent. Crosslinked dextrans are stable in salt solutions, organic solvents, and alkaline and weakly acidic conditions. Hydroxypropylation of a crosslinked dextran with a narrow pore size produces useful sorbents for the purification of low molecular mass organic compounds by size-exclusion and adsorption chromatography [4,27]. The most popular sorbent for this type of application, Sephadex LH-20, is solvated by weak and medium polarity organic

solvents. The maximum sample load is about 300 mg / g of dry sorbent, and the maximum exclusion limit about 4,000 Da.

Conventionally produced cellulose powders (microcrystalline cellulose) consist of irregularly shaped fibrous particles of limited use for column chromatography. Beaded cellulose is prepared by dissolution of cellulose powder in a suitable solvent, followed by droplet formation in a suspension medium, and subsequent solvent extraction or crosslinking. Cellulose triacetate and tricarbamate derivatives are useful as low-cost sorbents for the process-scale separation of enantiomers (section 10.4.2).

Synthetic organic gels in a range of particle and pore sizes are prepared by suspension polymerization for size-exclusion chromatography (section 4.2.4) [113-116,118,119]. Gels with a range of solvent compatibility and swelling properties are obtained by varying the identity of the monomers and their relative concentration. Classic poly(styrene-divinylbenzene) gels are suitable for the purification of synthetic polymers and fractionation of low molecular mass organic compounds. Poly(acrylamide)-type gels are generally superior for the purification of biopolymers, and include poly(acrylamide), poly(methyl methacrylate), poly(hydroxymethyl methacrylate), poly(tris[hydroxymethyl] methacrylate), poly(ethylene glycol dimethacrylate), poly(vinyl acetate, and poly(vinyl alcohol). Poly(acrylamide)-type gels are stable over the pH range 4-13.

The natural and synthetic polymer gels, described above, are suitable for the preparation of ion exchangers [6,7,113-115]. Simple coupling chemistry allows the introduction of sulfate, sulfonate (e.g. sulfoethyl, sulfopropyl and sulfobutyl), phosphoesters, and carboxymethyl groups. Cation exchangers are represented by tertiary amino groups (e.g. dimethylaminoethyl, diethylaminoethyl) and quaternary amino groups (e.g. tetramethylaminoethyl, tetraethylaminoethyl). The functional groups are attached to the polymer backbone through short, medium, or long spacer arms, by means of ether, alkylamine, or amide bonds. The diverse chemical nature of the support matrices, and the coupling chemistry employed results in ion-exchangers that differ significantly in protein retention and recovery [120-125]. Selection of a particular gel for a given application remains a difficult problem. The large number of sorbents now available, however, ensures a high level of success, if only after a series of screening experiments to eliminate gels with less desirable properties.

Amongst the genre of gel-based ion exchangers, are two types designed for improved performance in preparative-scale chromatography. Hyper-diffusive particles are described later (section 11.3.7.2). Tentacle ion-exchangers consist of linear polymeric chains anchored to the support matrix, Figure 11.10 [122,125,126-128]. The average length of the polymer chains is 15 to 50 monomer units, each monomer unit carrying a charge center. A fully extended tentacle chain is about 10 nm long. To provide uninhibited access of proteins to the interior of the gel matrix, large pore diameters (100 to 500 nm) are required. The flexibility of tentacle ion-exchangers allows the charge distribution to adapt to the protein structure, and is less likely to cause unfolding or denaturation of unstable proteins compared with conventional ion exchangers with a rigid array of binding sites. In addition, the binding capacity of

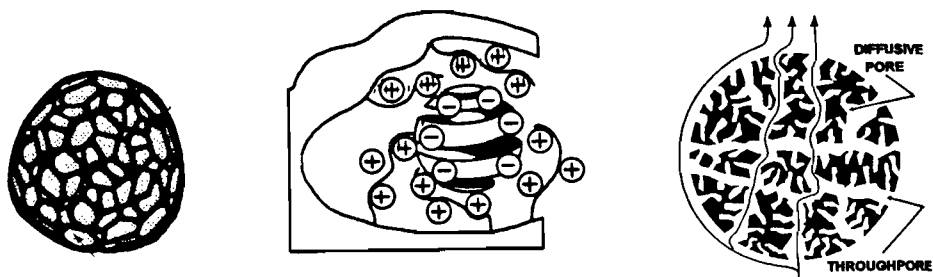


Figure 11.10. Schematic diagram of a hyper-diffusive (gel-filled gigaporous) particle (left), tentacle ion-exchanger interacting with a globular protein (middle), and a perfusive (diffusion-convection) particle (right).

tentacle ion exchangers is often greater than for conventional polymer gels. This is a result of the larger number of ionic sites on the tentacles, and the flexibility of the tentacle chains, which allows greater access to sites on the protein, especially for large proteins. In specific cases, tentacle ion exchangers provide a reduction of non-specific interactions with the gel matrix, and a general improvement in selectivity and band shape. The concept of tentacle media also finds use in the development of materials for hydrophobic interaction and affinity chromatography.

Hydroxyapatite is a crystalline form of hydroxylated calcium phosphate [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$] used as a powder, porous ceramic, or microcrystals embedded in an agarose matrix for the purification of proteins and DNA [129-131]. Hydroxyapatite provides complementary selectivity to conventional ion exchangers, and is often used for the further purification of fractions isolated by conventional ion exchangers. Separations on hydroxyapatite are due to a charged-based, mixed-mode retention mechanism. The surface of hydroxyapatite consists of positive calcium ions, referred to as C-sites, and clusters of negatively charged phosphate groups, referred to as P-sites, arranged in a crystalline structure. Carboxylic acid and phosphate functional groups are adsorbed at C-sites by coordination complexation. Ionized amine groups are adsorbed at P-sites. Continuous or stepwise concentration gradients of sodium or potassium phosphate at close to neutral pH, are used for separations. The phosphate ion competing with the protein or nucleic acid for the two types of adsorption sites. Adsorption of metal ions, particularly manganese, iron, and aluminum, from process materials results in discoloration of the hydroxyapatite and possible loss of separation properties [131].

11.3.7.2 Diffusion-Convection Sorbents

Diffusion-convection sorbents are biporous materials with a network of wide pores providing convective transport connected to smaller diffusion pores that possess most of the surface area, and are responsible for retention [132-136]. An optimized arrangement of diffusion and convection pores results in enhanced mass transfer properties and shorter separation times for macromolecules. Sorbents in this category include superporous agarose beads [137,138], biporous monolithic or continuous bed

columns (section 4.2.7) [49-51], and perfusive particles [139-143]. Superporous agarose beads are prepared by a double emulsification procedure forming large flow pores (superpores) with a diameter 0.25 to 0.05 of the particle diameter (106-180 μm) connected to normal diffusion pores. Perfusive particles are prepared by suspension polymerization, as rigid beads of highly crosslinked poly(styrene-divinylbenzene) made up of agglomerated microspheres, Figure 11.10. The void spaces between the microspheres and agglomerates define the pore size distribution, with the wide pores as regions separating the agglomerates. Particles of 20 or 50 μm diameter with convection pores of 0.6-0.8 μm and diffusion pores 0.08-0.15 μm are typically used for purification of biopolymers. Poly(styrene-divinylbenzene) particles can be operated at pressure (≤ 200 atm) without compression. However, these matrices cannot be used for protein purification because of strong and irreversible interactions of proteins with the hydrophobic sorbent surface. A more suitable hydrophilic surface, for example, can be achieved by coating the surface of the poly(styrene-divinylbenzene) beads with poly(vinyl alcohol), and subsequently crosslinking the poly(vinyl alcohol) with glutaraldehyde [141]. In practice, a wide range of functionalized coatings and biospecific ligands (antibodies, proteins, peptides, nucleic acids, carbohydrates, etc.) can be immobilized on the interior particle surface for separations by reversed phase, ion exchange, hydrophobic interaction, and affinity chromatography.

Transport of molecules through a homogeneous porous particle occurs by diffusion. For macromolecules, this is a slow process producing characteristic broad bands. At high flow rates diffusion-convection media allow convective mass transfer into the particle interior. Since convective transport of biopolymers is several orders of magnitude faster than diffusion, the interior binding sites within the pore network are contacted more rapidly than for packing materials relying solely on diffusive transport. Only a small percentage of flow transecting the particles is required in order to achieve a dramatic increase in mass transfer rates. The ratio of particle permeability to packed-bed permeability, or the split ratio, is an important parameter determining column performance. The column plate height and binding capacity is only weakly dependent on flow rate. The reduced residence time improves recovery of bioactive compounds and reduces the cost of large-scale purification processes.

Diffusion-convection particles have performance advantages over particles with standard pore sizes for the separation of macromolecules, mainly for applications that do not require high resolving power to accomplish fast separations. The low surface area of the particles, however, restricts the ultimate production rate. Polymeric gels have high loading capacities, but a weak mechanical structure, which restricts the optimum use of pressure and the production rate. For ion exchange purification of biopolymers, hyper-diffusive particles (also known as gel-in-a-shell or gel-filled gigaporous particles) overcome these limitations. This is done by encapsulating an ionic polymer gel in a rigid, porous silica-polymer composite shell, Figure 11.10 [10,123,141,144-148]. The pore volume of a silica substrate with surface reactive groups is filled with the desired ionic monomers, which are then polymerized within and throughout the pore volume. The gel structure is optimized with respect to the concentration of ionic groups, polymer

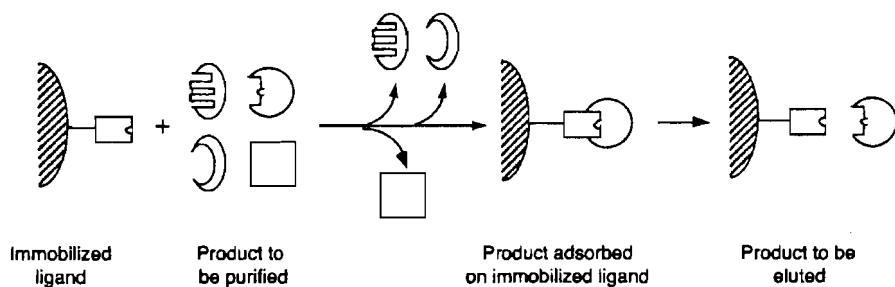


Figure 11.11. Principles of affinity chromatography. (From ref. [178]; ©Elsevier).

flexibility, and diffusive properties of the polymeric network. The shell is optimized with respect to particle size, pore size distribution, and mechanical strength. The high efficiency of the hyper-diffusive particles is attributed to the favorable permeability of the polymer gel and modest particle size (40-60 μm). The higher production rate results from the greater loading capacity of the hyper-diffusive particles compared with diffusion-convection particles.

11.3.8 Affinity Chromatography

Affinity chromatography is a well-established technique for the isolation and purification of macromolecules, generally proteins and polynucleotides, based on highly specific three-dimensional molecular recognition [6,10,113-116,149-152]. There are virtually no direct applications to the purification of low molecular mass organic compounds with the exception of a few micropreparative applications employing molecularly imprinted polymers, usually for the separation of enantiomers [153-158]. Even in this case, these materials are generally used for analysis. They are somewhat limited for preparative-scale chromatography by the heterogeneity of binding sites, poor efficiency, and low loading capacity. Improvements in synthetic design may eventually reveal their true potential for large-scale purification. In addition, the well-developed, small-scale applications of affinity chromatography to clinical diagnostics, protein binding studies, and solid-phase extraction, etc., are not considered in this section [159-162].

In affinity chromatography, a ligand (molecule having specific recognition capability) is immobilized on a suitable insoluble support (matrix), which is usually a natural or synthetic polymer gel in bead or membrane form. The analyte (or target) is adsorbed selectively (captured) by the immobilized ligand, by simply passing a solution of the analyte through the column under favorable conditions for analyte-ligand binding, Figure 11.11. Weak and non-specifically bound impurities are removed by rinsing the column with the buffer used for sample application, or other composition that does not displace the analyte. The analyte is recovered as a concentrated solution by elution under conditions that favor dissociation of the analyte-ligand complex. For example, by adjusting the pH, ionic strength, or temperature of the sample application buffer, or by using specific solvents, or competitive free ligands added to the sample buffer.

Table 11.6

Typical affinity ligands for protein separations

Ligand	Analytes
Metal ions	Proteins with an abundance of histidine, tryptophan, and cysteine residues
Reactive dyes	Most proteins, particularly nucleotide-binding proteins
Lectins	Glycoproteins, cells
Phenylboronic acids	Glycoproteins
Carbohydrates	Lectins
Nucleic acids	Nucleases, polymerases, and other DNA and RNA binding proteins
Amino acids	Proteases
Heparin	Plasma proteins, such as clotting factors
Nucleotides, cofactors,	Enzymes, substrates, and inhibitors
Proteins A and G	Immunoglobulins
Hormones, drugs	Receptor proteins
Antibodies	Antigens
Antigens	Antibodies

Binding of the analyte is a monolayer adsorption process characterized by an equilibrium isotherm, typically of the Langmuir or Freundlich type (section 1.5.5). The saturation capacity of the adsorbent can be estimated through these relationships. The capacity of the adsorbent is fully utilized when the column is saturated, corresponding to the breakthrough point for sample loading. Thus, the general conditions for elution chromatography are of limited application for modeling the separation process. Plate theory does not really apply, and slow kinetics of the adsorption/desorption process is generally more important.

Almost any compound may be used as an affinity ligand, if it binds selectively and reversibly to the analyte in question, and can be immobilized on a solid support without impairing the analyte-ligand-binding interaction, Table 11.6. For affinity chromatography the optimum dissociation constant for the ligand-analyte complex is about $10^{-4} - 10^{-8}$ M. Larger binding constants have the potential to deliver higher purification, but may prevent elution of the bound protein in its native state. Similarly, weaker binding constants are disadvantageous, since proteins are merely retarded and quickly breakthrough the column. Typical ligands used for preparative-scale chromatography are either group-specific or highly selective, in their binding properties. Group-specific ligands retain a range of analytes with similar binding properties. Highly selective ligands retain a single or small number of analytes.

For every protein purification problem there is always an affinity solution, but cost and safety considerations may render these solutions impractical. As an example, antibodies are widely used for analysis, where only relatively small amounts are usually required, but their production and purification on a large scale for preparative-scale chromatography may be difficult to justify economically. In some cases, Hhybridoma technology may be able to address this problem. Even if production costs are acceptable, the immobilized antibodies may be unstable over the sequence of sample application, elution, and sanitation required for multiple use of the affinity adsorbent. For these reasons, while biological ligands (antibodies, enzymes, receptors, lectin,

nucleic acids, etc.) continue to be used, their wider application is hampered by the above factors [149,150,163-166]. These considerations have led to increasing interest in low molecular mass synthetic ligands, such as reactive dyes (section 11.3.8.1), metal chelates (section 11.3.8.2), thiophilic ligands [167], and boronic acids [6,166,168] for preparative-scale affinity chromatography. Synthetic ligands are often group-specific, and improving the selectivity of separations is of great importance. Possibilities include using competitive ligands or displacers, or by designing new ligands tailor-made for the analyte of interest.

The support is expected to function as a scaffold covered with an optimum ligand concentration, in such a way that adequate access and orientation of the ligand for binding with the analyte is preserved. Desirable properties in an ideal support include: an absence of non-selective binding interactions associated with charged and hydrophobic surface groups; an abundance of surface functional groups (hydroxyl, carboxylic, amide, etc.) for ligand immobilization; adequate mechanical and chemical stability under a wide range of conditions, such as high and low pH, and temperature, and in situations that require organic solvents, detergents, etc. The use of modest pressures and the requirement of high surface areas to maximize sample capacity, favor the use of spherical porous particles with narrow particle size ranges between 10 to 500 μm and pore sizes between 10-500 nm. Crosslinked and beaded poly(saccharides), particularly agarose and to a lesser extent cellulose, synthetic macroporous polymers, particularly poly(acrylamide)-based gels, and inorganic oxides (coated silica, porous glass), as well as hyper-diffusive and convection-diffusion particles (section 11.3.7.2) are widely used [113-116]. Crosslinked beaded agarose with an exclusion limit of about 10^7 Da is the most popular support for laboratory-scale applications.

Reagents that facilitate either direct reaction between the ligand and support, or introduce a different reactive functional group at the support surface, which is subsequently reacted with the ligand, are widely employed for immobilization of affinity ligands [6,113,169-173]. The majority of reactions for ligand immobilization employ activation of hydroxyl groups by reagents containing halogen, epoxy or carbonyl functional groups, Table 11.7. These supports are then reacted with ligands containing nucleophilic groups, such as amine, hydroxyl, or thiol groups. Since virtually all proteins and the majority of synthetic ligands contain at least one of these functional groups (or these functional groups are easily introduced into suitable ligands), a wide range of coupling chemistries is available for optimizing the attachment of the ligand to the support. Other reactions, employing carboxylic acid groups, for example, are available. The large number of chemically activated supports now available simplifies the production of affinity adsorbents in the laboratory.

To ensure minimum interference in the binding of the analyte by the immobilized ligand the coupling reaction between the ligand and support should occur through a region of the affinity ligand remote from the binding site. In addition, the binding site of biopolymers is often located deep within the three-dimensional structure of the molecule, and steric hindrance prevents interaction of the ligand with the analyte. In this case, spacer arms, usually short chains 6-12 units (CH_2 and/or O) long, are imposed

Table 11.7

Coupling reactions for supports containing surface hydroxyl groups

Activating agent	Reactive group on ligand
Cyanogen bromide	Primary amines
Tresyl chloride	Primary amines, thiols
Epichlorohydrin	Primary amines, thiols, hydroxyls
1,4-Butanediol diglycidyl ether	Primary amines, thiols, hydroxyls
1,1'-Carbonyldiimidazole	Primary amines, hydroxyls
Cyanuric chloride	Primary amines, hydroxyls, thiols
Divinylsulfone	Primary amines, hydroxyls
Sodium periodate	Primary amines
Glutaraldehyde	Primary amines

between the support surface and the ligand to ensure ligand accessibility to the binding site. In addition, it is generally recognized that there is an optimum concentration of immobilized ligand for purification of a specific protein. This concentration, however, varies with the protein in question, and the optimum loading has to be ascertained by experiment.

Affinity chromatography can be used as a single stage purification and concentration technique. More typically, though, it is used for one or more stages of a purification cascade that possibly includes ion exchange and hydrophobic interaction chromatography, as well as traditional isolation techniques, such as precipitation. Affinity chromatography is uniquely suited to the recovery of proteins from dilute solutions. Its high selectivity leads to its use for the final purification of isolates containing small amounts of impurities with similar properties to the desired product. How affinity chromatography is used in a purification cascade, however, will largely be dictated by economic considerations, since affinity adsorbents tend to be more expensive than other materials.

11.3.8.1 Reactive Dyes (Biomimetic Ligands)

While biological ligands display high selectivity, they suffer from low binding capacity, limited reusability, and low scale-up potential. Synthetic ligands offer a number of advantages for the purification of proteins. Affinity adsorbents prepared from synthetic ligands are less expensive, scalable, durable, and reusable over multiple cycles. The exceptional stability of synthetic adsorbents allows harsh elution, cleaning, and sterilization protocols to be used. Synthetic dyes are the most important alternative to natural ligands for preparative-scale affinity chromatography [6,174-179]. These dyes are structural mimics for naturally occurring nucleotides and flavins. The most important group of synthetic dyes contain reactive, usually chlorotriazine, groups that facilitate bonding to different supports. These dyes also possess an extended aromatic backbone with a variety of functional groups that allow simultaneous interactions with protein binding sites, mimicking natural ligands. The often-poor selectivity, variable composition, leakage, and toxicity of the triazine dyes, which are inexpensive commodity chemicals, limit their potential applications. As a template for the development of more specific ligands for protein purification, however, they

have proven uniquely useful. From molecular modeling techniques, it is possible to redesign the parent dye, or design *de novo* a new dye, that mimics naturally occurring ligands ("biomimetic" dye). The design strategy consists of several defined stages. Molecular modeling is used to formulate a preliminary design of a complementary ligand using a suitable binding region of the target protein as a template. Solid-phase synthesis of a limited near-neighbor combinatorial library allows optimization of the ligand-protein interaction. The most promising ligands are then synthesized on a larger scale to provide sufficient material to characterize key properties and to permit chromatographic evaluation. For the latter purpose, the ligand is immobilized on a suitable support, and the chromatographic conditions optimized for purification of the target protein from the sample matrix. It is the possibility of combining information from X-ray crystallographic or NMR protein structures with combinatorial synthesis and advanced computational tools, that make the rational design of affinity ligands feasible. Similar design strategies are used to prepare synthetic ligands based on peptides, oligonucleotides, or small proteins.

11.3.8.2 Immobilized Metal Ion Affinity Chromatography

Immobilized metal ion affinity chromatography (IMAC) relies on the ability of certain amino acid side chains to form coordinate bonds with immobilized metal ion complexes [6,181-186]. The adsorption of proteins mainly takes place through interactions with the imidazole ring of histidines. For other amino acids with electron donor atoms in their side chains, binding tends to be weak. Cysteines in natural proteins, are rarely available in an appropriate reduced form for binding to chelated metal ions.

Immobilized metal ion affinity chromatography provides group-specific separations in general, as well as a highly selective purification tool for target proteins from complex biological samples. It is one of the most popular methods for the purification of recombinant proteins modified with oligo-histidine affinity tags at the N- or C-terminus [187-189]. Since oligo-histidine residues are uncommon among natural proteins, such affinity handles allow a one-step isolation of tagged proteins in high purity. In most cases, the insertion of a small tag composed of (usually) six histidine residues hardly modifies the activity or stability of the protein. This method is proving increasingly popular for the low cost production of large quantities of pure enzymes for industrial applications, and for use in molecular biology. In addition, substantial progress has been made in the selective purification of phosphorylated proteins using immobilized iron (III) chelates [182,190]. The main practical attributes of immobilized metal ions for affinity chromatography are their stability to a wide range of operating conditions, high capacity, mild elution conditions, ease of regeneration, and low cost.

The adsorption of proteins in IMAC is based on the coordination between an immobilized metal ion and electron donor groups on the protein surface. Soft metal ions [e.g. Cu (II), Co (II), Zn (II), Ni (II)] show a preference for coordination with N-containing functional groups, such as the imidazole ring of histidine. Hard metal ions [e.g. Al (III), Ca (II), Fe (III)] show a preference for oxygen-containing groups, such as carboxylate or phosphate. The metal ions are immobilized on the support by interaction

with a chelating ligand, such as iminodiacetic acid (IDA), nitrilotriacetic acid (NTA), carboxymethylated aspartic acid (CM-Asp) or tris(carboxymethyl)ethylenediamine (TED). The binding of proteins to the affinity adsorbent depends on the choice of metal ion and the structure of the ligand (chelate group), as well as the length of any spacer arm, and the surface concentration of the ligand. All of which presupposes that the range of support pore sizes does not interfere with access of the protein to the metal binding site. Metal ion transfer must be avoided (the chelating ligand must bind the metal ion tightly enough to prevent its removal by sample proteins).

Protein adsorption by the affinity adsorbent requires a pH that favors chelation with the protein (e.g. the nitrogen atoms of the imidazole ring of histidine are unprotonated), normally in neutral or slightly basic medium. Relatively high ionic strength buffers (e.g. containing 0.1 to 1.0 M NaCl) to reduce nonspecific electrostatic interactions between the protein and the metal chelate complex. Once the sample is loaded on the affinity adsorbent, weakly bound proteins are removed by rinsing with several bed volumes of buffer, or more efficiently, by a low concentration of eluting agent. A rinse step with a low ionic strength buffer allows removal of proteins retained by hydrophobic interactions with the support. The target protein is recovered by elution with an appropriate pH buffer, ligand exchange, or extraction of the metal ion by a stronger chelating agent, such as EDTA. Elution buffers with a lower pH, or declining pH gradient, are widely used for eluting target proteins bound to soft metal ions. A further option is to use a displacer, such as imidazole. With hard metal ions, elution requires an increasing pH gradient, or addition of a competing reagent to the mobile phase, such as organic acid or phosphate. Immobilized metal ion affinity chromatography columns have excellent regeneration properties.

11.4 SUPERCRITICAL FLUID CHROMATOGRAPHY

Packed column supercritical fluid chromatography is emerging as a complementary technique to normal-phase liquid chromatography for the purification of low to intermediate molecular mass organic compounds. Favorable attributes include faster separations, less solvent waste, and rapid equilibration simplifying the use of composition gradients. There are no applications to biopolymers and other polar compounds with low solubility in supercritical fluid carbon dioxide and its mixtures with organic solvents. These mobile phases, sometimes containing small amounts of additives to improve peak shapes, are used exclusively in current applications. In addition, virtually all applications are limited to slurry packed semipreparative columns with internal diameters < 25 mm containing small diameter particles (< 20 μm) [191-198]. This is not due to any fundamental restrictions, but reflects the limited flow range of hybrid instruments designed for both analytical and preparative use, found in most laboratories. These instruments meet the demands of laboratories that require access to a preparative capability on an occasional basis, and are interested in purifying a modest amount of sample.

Instruments for all purposes from the laboratory-scale through pilot plant to process-scale, including simulated moving bed systems, are available [6,106,107,194,195,199-201]. Large-scale systems are designed with different engineering and economic considerations in mind compared with laboratory-scale systems, and are not discussed in this section. In the last few years, fully automated systems for high-throughput purification of combinatorial libraries with UV or mass-directed fraction collection have been described, completing the circle of laboratory-scale purification processes [202,203].

Method development is similar to the scale-up approach for high-pressure liquid chromatography (section 11.3.4.3). Preliminary analytical separations are used to optimize the selectivity factor and separation time. The sample concentration or volume is then increased to the maximum sample load for linear chromatography, or to overload conditions for increased productivity (section 11.3.4.4). Band broadening under column overload conditions is generally governed by saturation of the mobile phase [191]. Separations under overload conditions require injection of large sample volumes, unless the sample can be highly concentrated in a suitable solvent. For samples in weak solvents, injection volumes up to about 2 ml can be made on semipreparative columns. However, severe peak distortions and splitting can occur with even small injection volumes when the elution strength of the sample solvent is greater than the elution strength of the mobile phase (section 7.6.2.1). Strong solvents may be required to achieve adequate sample solubility. Solvent-elimination injection techniques are used to circumvent this problem [194,205,206]. An automated solvent-elimination injection system, uses two six-port valves separated by a small packed column trap. The sample is loaded onto the trap in the usual way and excess solvent evaporated by a stream of (warm) gas. The solvent-free sample is then transferred from the trap to the separation column by dissolution in the mobile phase. Otherwise, the sample is extracted from the trap with a mobile phase suitable for focusing the sample at the head of the separation column. Solventless injection is also useful for handling aqueous samples (although elimination of water is time consuming), and for concentrating dilute samples. For modest sample volumes, the evaporation step can be performed in an empty capillary tube, so long as there is a sufficient vapor pressure difference between the solvent and sample (see section 7.6.2.1).

Products are collected after detection and/or on a time basis by automated fraction collection using different approaches [194,202,203,207-210]. Decompression of the mobile phase occurs with cooling, phase separation of mobile phase mixtures, and expansion of the fluid phase to a gas. Possible problems for sample collection are low recovery due to precipitation of the sample in transfer lines, and formation of aerosol particles that escape the collection vessel. Simple collection devices like an empty vessel at atmospheric pressure, or collection in solvent by bubbling the expanded gas through the solvent, result in significant sample losses at the high flow rates associated with semipreparative columns. For carbon dioxide as the mobile phase, cyclone or solid-phase adsorption traps are suitable trapping devices. Cyclone traps are designed for isolating single products or sections from a chromatogram, and are unsuitable for collecting several fractions from repetitive injections. The capacity of sorbent traps is

limited by the amount and retentive properties of the sorbent, and recovery of the sample requires off-line solvent desorption.

For preparative-scale applications using mixtures of carbon dioxide and an organic solvent, high-pressure trapping and solvent trapping are the preferred methods. High-pressure trapping involves transfer of fractions to a pressurized vessel, and condensation of the sample at low temperature. Typically, the temperature is low enough to solidify the mobile phase. Subsequently, the pressure is slowly released, and the carbon dioxide evaporated, leaving the sample in modifier as solvent. For solvent trapping, an additional solvent flow of 5 to 40% of the mobile phase flow is added upstream from the pressure control valve and after the detector. The solvent flow maintains the sample in solution after depressurization of the mobile phase. The solvent enhanced mobile phase is collected in a vessel partially filled with a small amount of solvent. The product stream is introduced below the solvent level, and the carbon dioxide allowed to escape. The collection vessel is cooled to increase the collection efficiency.

11.5 GAS CHROMATOGRAPHY

The most common types of preparative-scale gas chromatographic instruments are based on packed column technology [211-217]. For simple separations, a short, wide packed column is generally used (e.g. 1-3 m x 6-10 cm I.D.) For difficult separations, higher column efficiencies are required, and long, narrow packed columns (e.g. 10-30 m x 0.5-1.5 cm I.D.), or multidimensional chromatographic techniques (section 3.8.1) [218], are used. Wide bore packed columns cannot be coiled, and unless a purpose designed preparative-scale gas chromatograph is available, the choice may be limited to long, narrow, coiled columns. Other critical considerations include the method of injection, detection, and sample collection. Syringe injection is possible for small volumes of volatile samples (e.g. 100 μ l) using slow injection. Typical sample volumes are 0.1 to 10.0 ml, which are too large for injection by conventional techniques developed for analytical packed columns. In this case, an automated injector employing pneumatic transfer from a reservoir through a capillary restrictor, a pneumatic piston pump, or syringe pump, is used. The injection process is controlled by time; a necessity owing to the limited thermal capacity of injection block heaters, and their inability to flash vaporize large sample volumes. To assist solvent vaporization, an evaporation device between the injector and column, is usually installed. This is often a tube, heated separately from the column oven, and packed with glass beads or metal spheres to increase its thermal capacity while simultaneously reducing dead volume effects. The evaporation unit is usually heated to a temperature 50°C above the boiling point of the least volatile sample component. Vaporization problems can be circumvented by slow, on-column injection without carrier gas flow. This is often the most practical solution for injecting large sample volumes using analytical instruments. The principal disadvantage is the possibility of stripping the stationary phase from the head of the column. For on-column injection of liquid samples, a substantial pressure change may result from

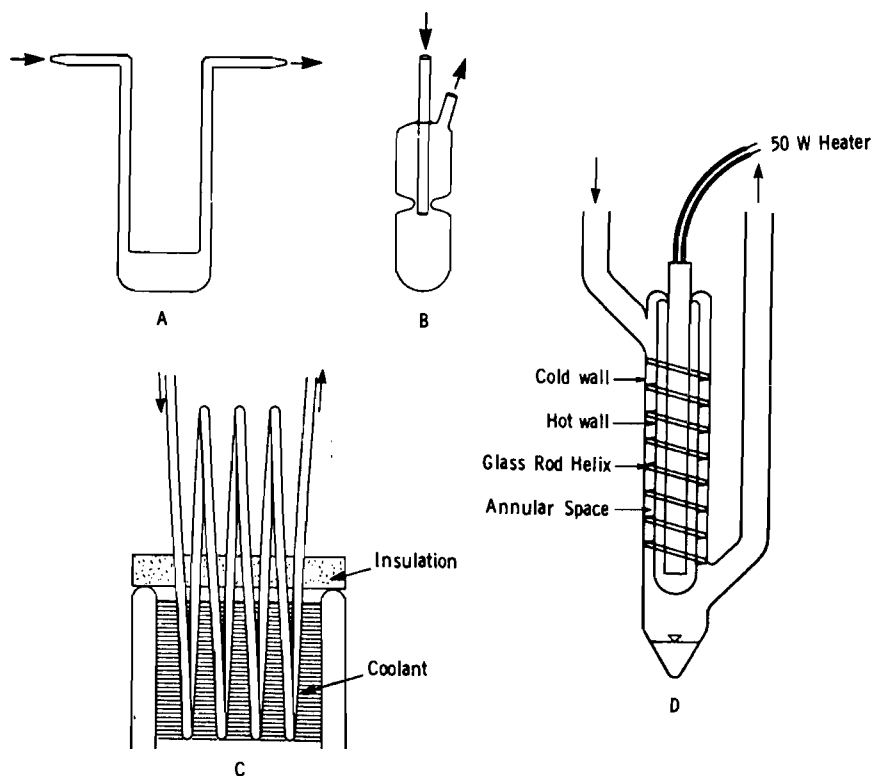


Figure 11.12. Traps for sample recovery from a gas chromatographic effluent. A, U-tube; B, simple trap; C, multiple temperature gradient trap; D, Volman trap.

sample vaporization. Backflushing of sample into the carrier gas lines can be a problem, solved by positioning a backflush or needle valve in the carrier gas line.

Separations are usually performed isothermally. Temperature programming is impossible with wide bore columns owing to the uneven radial temperature gradients generated across the column diameter. Temperature programming is only possible with long, narrow bore columns, and then only slowly. Separated components are detected by a thermal conductivity or flame ionization detector connected to the column via a splitter, so that only a few percent of the total column flow is diverted to the detector.

Collection of sample vapors in the carrier gas effluent is performed automatically or manually, initiated by the detector signal. In purpose-designed instruments, injection and sample collection are automated for unattended operation. The exit from the detector splitter is usually led out through a side wall of the oven and thermostatted to avoid condensation of the sample. Several methods are used to trap samples, including packed and unpacked cold traps, solution and entrainment traps, total effluent and adsorption traps, Volman traps, and electrostatic precipitators, Figure 11.12 [219-223].

The minimum requirement for efficient sample collection is that the partial pressure of the solute entering the trap must be greater than its equilibrium vapor pressure with the condensed phase at the trap temperature. The equilibrium vapor pressure of the solute can be reduced by lowering the temperature, by dissolving the solute in a solvent, or by adsorbing it onto a material of high surface area. However, the most common approach is to reduce the temperature of the effluent, either in a trap filled with a material such as glass beads or column packing, or in a simple open tube trap. In practice, the residence time of the sample in the trap may be too short to reach equilibrium. In which case, the sample will tend to pass directly through the trap, either as a supersaturated vapor or as a fog composed of small liquid droplets. Cold trapping of samples with boiling points greater than about 150°C is almost inevitably accompanied by the formation of aerosols, which are then swept through the trap. Cooling the effluent through a gradual temperature gradient rather than subjecting it to an abrupt temperature change can minimize this effect. In addition, turbulent flow within the trap facilitates the collision of liquid droplets with the wall, thereby inducing their retention. The Volman trap consists of a double-walled vessel within which turbulence is created by maintaining the walls at different temperatures. The Volman trap is particularly useful for recovering gram-scale quantities. Electrostatic precipitators are also used to trap large fractions. As aerosols contain large numbers of charged droplets, the trapping efficiency is improved by passing the cooled effluent through a large electrostatic field. For occasional sample trapping, a simple packed or empty glass U-tube cooled in a dry ice-acetone or liquid nitrogen coolant, usually suffices.

The stationary phase for the separation is identified from preliminary analytical separations. The support material is selected with the column length in mind. It is normally coarse with a narrow size distribution (e.g. 35-40 or 80-100 mesh). At typical carrier gas flow rates of 100 to 1000 ml/min, depending on the column internal diameter, coarse packing is needed to allow operation at reasonable inlet pressures. Since the stationary phase loading is generally higher and column length longer than is typical for analytical separations, operation at higher temperatures is also common.

Reproducibly packing columns with reasonable efficiency is more difficult for wider bore columns [212,224-226]. This inferior performance is due to the uneven radial packing density resulting from particle size segregation, with the larger particles being closer to the wall. In addition, the packing in this region is less dense, due to the physical constraint of the wall. To minimize this problem, the shake, turn, and pressure method is recommended [224]. The column is shaken in the radial direction and rotated along its long axis while being packed, and periodically pressurized. Packing under suction with vertical tamping, affords a faster approach [226]. Since column permeability is an important consideration in preparative-scale gas chromatography, it is essential to minimize the production of fines during the packing process.

Open tubular columns have a lower sample capacity than packed columns, but are useful for isolating a few milligrams of components from complex mixtures under high-resolution conditions [227-232]. Automated sequencing of injection and fraction collection to accumulate sufficient sample is generally used. Short lengths of wide bore

capillary tubes, coated with a thick film of stationary phase, are efficient traps for room temperature sample collection. Either solvent or thermal desorption is used for sample recovery. Because of narrow peak widths, rapid switching between traps and/or waste is required to avoid cross contamination.

11.6 COUNTERCURRENT CHROMATOGRAPHY

Countercurrent chromatography is a preparative-scale technique, using two mutually saturated immiscible liquid phases in an open column for separations governed by the difference in partition coefficients for sample components [6,233-237]. One phase (the mobile phase) continuously passes through the other (the stationary phase), which is permanently retained in the column by an effective combination of column configuration and centrifugal force. The main advantages of countercurrent chromatography compared with column liquid chromatography are the absence of a solid support, and a higher loading capacity for columns of equal volume. Laboratory-scale instruments with a column volume of 100-400 ml can handle sample sizes of 0.5-10 g per injection. Process-scale machines are capable of handling larger sample sizes, but there is little information available concerning the design and operation of these instruments [238,239]. The major use of countercurrent chromatography is for the isolation of labile natural products, and to a lesser extent, the extraction and purification of pharmaceutical compounds, biopolymers, metal ions, and industrial chemicals [3-6,234-237,240].

All countercurrent chromatographs are either hydrostatic or hydrodynamic equilibrium systems [233,235,241,242]. Several early versions of these chromatographs became essentially redundant with the development of the high-speed countercurrent chromatograph [6,235,236], and the centrifugal partition chromatograph [234,243]. The high-speed countercurrent chromatograph (HSCCC) is an example of a hydrodynamic equilibrium system, Figure 11.13. A cylindrical column holder is equipped with a gear that is coupled to an identical stationary gear mounted at the central axis of a centrifuge. This gear arrangement produces a synchronous planetary motion of the column holder (i.e. revolution around the central axis of the centrifuge and rotation about its own axis), both at the same angular velocity, and in the same direction. This planetary motion prevents the tubes introducing and receiving the mobile phase from twisting, allowing continuous elution through the rotating column without the use of a rotary seal device. The column is directly wound around the holder to form single or multiple coiled layers. The sample is introduced from either end of the column, or at its midpoint.

The motion of the column generates an Archimedian screw force. Independent of density, the contents of the column are driven towards one end called the head (the other end is referred to as the tail) [244]. The forces acting on the coiled column cause vigorous mixing of the two phases with formation of a succession of mixing and demixing zones in each successive turn of the coil. When hydrodynamic equilibrium is established, the phase ratio is constant, and only the mobile phase moves through the

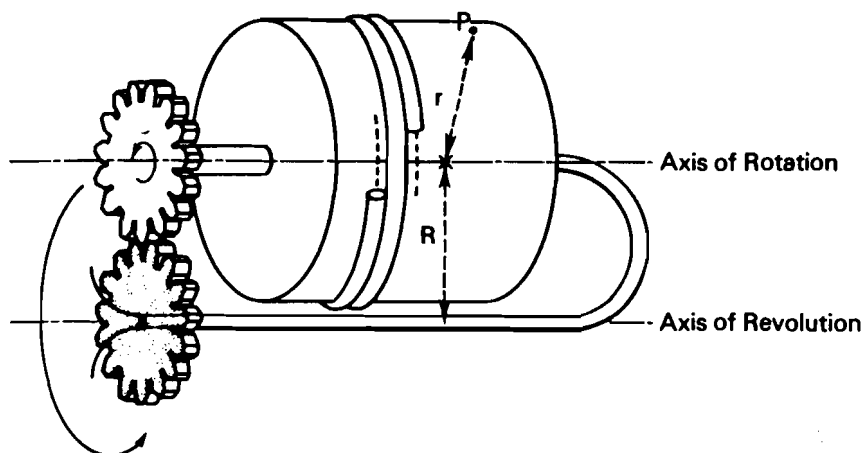


Figure 11.13. Design principle of the multilayer coil planet centrifuge for high-speed countercurrent chromatography. (From ref. [235]. ©John Wiley & Sons)

column. The velocity of the mobile phase is a linear combination of the flow rate and the rotation speed [245]. The stationary phase is stable only as long as the centrifugal field is applied [246]. There is a minimum flow rate at which the stationary phase is forced out of the column. For the same operating conditions, flow rates about 5% of this value are desirable for stable operation and ease of sample introduction. For flow rates greater than 10% of the minimum displacement flow rate, the stationary phase is only weakly retained, and sample injection may result in stationary phase loss.

Centrifugal partition chromatographs employ a complex arrangements of interconnected channels and narrow ducts in a centrifugal force field to promote mixing and separation of the two liquid phases, Figure 11.14. The column consists of a number of serially connected channels engraved in a plastic plate. Several plates are put together to form a cartridge attached to the rotor of a centrifuge. The column length is variable, and depends on the number of connected cartridges. The liquid stationary phase is retained in the channels and the mobile phase, which can be either the denser or lighter solvent phase, passes through it and collects in the ducts. At present, it is uncertain how the mobile phase actually flows through the stationary phase. While in the channels, the mobile phase may be continuous, like a film against one of the walls, flowing very quickly, then accumulating in a pool, before transfer to the next channel through a duct. Alternatively, it may be discontinuous, falling as droplets in the gravitational field, which are transferred immediately to the duct upon arriving at the channel outlet. A significant pressure drop is generated over the column during normal operation of the chromatograph. The mobile phase enters and leaves the column via rotary seals, which limit the maximum operating pressure to about 60 atm. at typical rotational speeds of 500-2000 rpm. Pressure constraints restrict the number of usable solvent systems, because flow rates that are too slow result in low sample throughput and poor separation

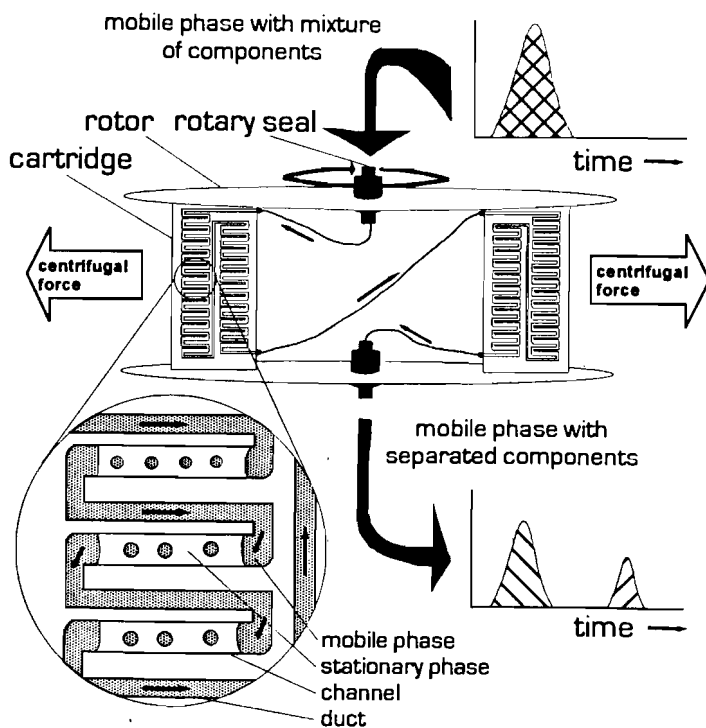


Figure 11.14. Design principle of the centrifugal partition chromatograph. (From ref. [234]. ©Marcel Dekker).

efficiency. Visualization of the flow patterns in the centrifugal partition chromatograph indicate that better phase mixing leads to improved separation efficiency, and confirm that the key parameters acting on mixing are flow rate and rotational speed [243].

Comparing the two types of countercurrent chromatograph, those working largely on hydrostatic principles can retain almost any biphasic system, but promote less efficient mixing resulting in low separation performance (wide peaks). Hydrodynamic systems provide better mixing and typical column plate numbers of 300-1000, but stationary phase retention is not as good. Some solvent systems used in the centrifugal partition chromatograph may prove unstable in the high-speed countercurrent chromatograph.

Preparative-scale separations are optimized on an analytical scale initially to minimize sample waste, and then scaled-up to a large volume column. A series of screening experiments are employed to identify the most selective solvent system for the separation in which the sample has favorable solubility [6,237,245,247]. The solubility criterion is important for preparative-scale separations, since the sample throughput is limited by the phase in which the sample is least soluble. In addition, the solvent system should provide a suitable range of partition coefficients for the target compounds, and

Table 11.8

Two-phase solvent systems for countercurrent chromatography

Solvent system	Composition (volume basis)
Hexane-acetonitrile	1:1 (wide range of compositions possible)
Hexane-methanol	1:1 (wide range of compositions possible)
Hexane-acetonitrile-chloroform	5:5:1
Hexane-ethanol-water	6:5:1
Hexane-ethyl acetate-acetonitrile-methanol	5:2:5:4
Hexane-ethyl acetate-methanol-water	3:7:5:5 or 1:1:1:1
Chloroform-methanol-water	7:13:8 or 1:1:1 or 13:7:2 or 4:4:3 or 5:5:3
Toluene-acetonitrile-water-ethanol	3:4:3:2
Chloroform-methanol-0.2 M acetic acid	1:1:1
Ethyl acetate-ethanol-water	2:1:2
n-Butanol-water	1:1 (wide range of compositions possible)
n-Butanol-acetic acid-water	4:1:5
n-Butanol-ethyl acetate-water	4:1:4

the solvent mixture selected as the stationary phase should be adequately retained in the column under the chosen operating conditions, Table 11.8. The partition coefficients are easily estimated from the ratio of the sample concentration in each phase of a small volume of the biphasic solvent system at equilibrium using UV absorption or thin-layer chromatography. In general, the most suitable range of partition coefficients, K , is $1 < K < 2$ for the centrifugal partition chromatograph and $0.5 < K < 1$ for the high-speed countercurrent chromatograph. Once the partition coefficient is established, the retention volume for each solute in the separation system is given by

$$V_R = V_{MP} + K(V_T - V_{MP}) \quad (11.2)$$

where V_R is the solute retention volume, V_{MP} the volume of mobile phase in the column (estimated as the retention volume of an unretained solute) and V_T the total column volume. The partition coefficient is defined as the ratio of the solute concentration in the stationary phase divided by its concentration in the mobile phase at equilibrium. Since either phase of a two-phase system can be used as the mobile or stationary phase, the choice of operating conditions affects the value of the retention volume in Eq. (11.2).

To increase the loading capacity of ionizable compounds, pH-zone refining countercurrent chromatography is used [6,235,248,249]. This technique produces a train of highly concentrated rectangular zones, similar to those obtained in displacement chromatography (section 11.3.5). The method requires the presence of a retainer acid or base in the stationary phase and a suitable counterion in the mobile phase. An example of a typical system consists of methyl *tert*-butyl ether containing 0.05 M trifluoroacetic acid (retainer acid) as the stationary phase and water containing 0.015 M ammonia (pH = 10) as the mobile phase for the separation of organic acids. Zone sharpening is a result of the recycling of the organic acids between the two phases, where they are alternately ionized and neutralized. The result of these exchanges is the formation of a series of solute zones with sharp boundaries moving through the column at the same speed. Each zone

consists of a single compound with its own specific pH. The pK_a and hydrophobicity of each compound determine the elution order of the zones. Charged minor components are concentrated at the boundaries of the major zones and elute as sharp peaks. The method is also applicable to the preparative-scale separation of enantiomers using an ionic chiral selector in the stationary phase.

The advantages of pH-zone refining for preparative-scale applications are an increased sample loading capacity for a given column (ten fold or more), the production of highly concentrated fractions, the concentration of minor components making them easier to detect, and the possibility of detecting compounds without a chromophore by monitoring pH. For compounds with similar hydrophobicity a minimum difference of 0.2 pK_a units is required. The method is not suitable for trace analysis, and each sample component should be present at > 0.1 mM.

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Subject Index

- AC = affinity chromatography
 - CCC = countercurrent chromatography
 - CE = capillary electrophoresis
 - CEC = capillary electrochromatography
 - CGE = capillary gel electrophoresis
 - CIEF = capillary isoelectric focusing
 - CITP = capillary isotachopheresis
 - FTIR = Fourier transform infrared spectroscopy
 - GC = gas chromatography
 - IEC = ion-exchange chromatography
 - IPC = ion-pair chromatography
 - LC = liquid chromatography
 - LLE = liquid-liquid extraction
 - LSC = liquid-solid chromatography
 - MEKC = micellar electrokinetic chromatography
 - MS = mass spectrometry
 - NMR = nuclear magnetic resonance spectroscopy
 - OTC = open tubular column
 - PC = packed column
 - RPC = reversed-phase chromatography
 - SEC = size-exclusion chromatography
 - SFC = supercritical fluid chromatography
 - SFE = supercritical fluid extraction
 - SPE = solid-phase extraction
 - TLC = thin-layer chromatography
 - QSAR = quantitative structure-activity relationships
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